Inflammation & stimulation

INFLAMMATION & STIMULATION

Layout: Tiny Wouters
Cover: © Karin Kuhlmann, Inflammable Matter, 2003, c/o Pictoright Amsterdam 2012
Production: CPI – Wöhrmann Print Service , Zutphen

ISBN: 978-94-6203-251-4

The printing of this thesis was financially supported by Nationaal Epilepsie Fonds, Cyberonics, Eisai BV, UCB Pharma B.V., School for Mental Health and Neuroscience and Kempenhaeghe.

INFLAMMATION & STIMULATION

PROEFSCHRIFT

Ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. dr. L.L.G. Soete,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op vrijdag 7 december 2012 om 12:00 uur

door

Marlien Wilhelmina Aalbers

Promotores:

Prof. dr. J.S.H. Vles
Prof. dr. M.H.V. de Baets

Copromotores

Dr. G. Hoogland
Dr. H.J.M. Majoie

Beoordelingscommissie:

Prof. dr. R.J. van Oostenbrugge, voorzitter
Prof. dr. H.A. Drexhage (Erasmus MC)
Prof. dr. L. Lagae (UZ Leuven)
Prof. dr. F.C.S. Ramaekers
Prof. dr. Y. Temel

Aan mijn ouders

Contents

Chapter 1

General introduction

Epilepsy

Epilepsy is one of the most common neurological disorders affecting up to 1% of the worldwide population (Sander, 2003). The first reports on epilepsy date back to 2000 BC, when the disorder was considered a divine malady or demonic possession (Magiorkinis, et al., 2010). Although epilepsy was already acknowledged as a brain disorder in the Hippocratic writings on the 'Sacred disease' (400 BC), for ages the general belief persisted that epilepsy had some kind of supernatural cause (Eadie, 1995). Even today, many patients suffer from stigma and exclusion in different parts of the world (de Boer, 2010).

Epilepsy is an umbrella term for a group of disorders with various clinical manifestations and underlying aetiologies that are all characterized by the occurrence of spontaneous seizures. A seizure is "a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain'' (Fisher, et al., 2005). The symptoms strongly depend on the intensity, localization, and spread of abnormal neuronal activity, and symptoms can therefore vary from patient to patient and from seizure to seizure. On the basis of the localization of abnormal activity, seizures can be classified as focal or generalized: during focal seizures the epileptic activity originates within networks limited to one hemisphere, while during generalized seizures activity arises in and engages bilateral networks (Berg, et al., 2010).

Specific epilepsy syndromes can be defined on the basis of the combination of clinical features, signs, and symptoms. One of these is temporal lobe epilepsy, the most common type of human epilepsy characterized by seizure onset in the medial temporal lobe.

Inflammation in epilepsy

The pathophysiology of epilepsy is not fully elucidated. One of the processes that might contribute to the transition from a normal brain into an epileptic one is inflammation. A first indication that hinted at a possible role for inflammation in epilepsy was the finding that several anti-inflammatory treatments had anticonvulsive effects in patients that were resistant to antiepileptic drugs (Ozkara and Vigevano, 2011). Moreover, encephalitis can cause epilepsy, for example in Rasmussen encephalitis, a severe epileptic childhood encephalopathy with focal seizures (Bien, et al., 2007, Vincent, et al., 2010). Furthermore, many autoimmune diseases are associated with the occurrence of seizures and there is an association between epilepsy and several specific antibodies such as antibodies against one of the glutamate receptors, the N-methyl-D-aspartate receptor (NMDAR) (Billiau, et al., 2005, Dalmau, et al., 2008, Vincent, et al., 2010).

Inflammation might not only be relevant for seizure disorders with a direct infectious or immune mediated aetiology, but also in other seizure disorders. Increased levels of pro-inflammatory cytokines have been demonstrated in patients with different epilepsy syndromes and various animal models. However, it is unclear whether these cytokine changes are intrinsic to epilepsy itself or whether they are related to the underlying pathology. Therefore, we aimed to study cytokine changes in patients suffering from temporal lobe epilepsy with and without neuronal cell death, i.e. with and without hippocampal sclerosis. Furthermore, we evaluated cytokine levels in the amygdala-kindled rat, an animal model associated with little neuronal cell loss (Morimoto, et al., 2004, Tuunanen and Pitkanen, 2000), in order to determine the influence of neuronal cell loss on inflammation. In both studies we aimed to determine a broad array of different cytokines simultaneously because the outcome of an inflammatory reaction is determined by the interplay between different cytokines.
Cytokines can contribute to seizure generation. For example, application of the pro-inflammatory cytokine interleukin-1β (IL-1β) lowers the seizure threshold and increases seizure severity and seizure duration in animal models (Vezzani, et al., 1999). One of the mechanisms through which inflammatory molecules can influence excitability is by phosphorylation of the NMDAR. For instance, the binding of IL-1β to its type I receptor leads to phosphorylation of the NMDAR (Viviani, et al., 2003). In this way IL-1β facilitates the influx of calcium via NMDAR-mediated ion channels. This increases neuronal excitability, which leads to increased seizure susceptibility (Balosso, et al., 2008). Activation and phosphorylation of the NMDAR was proven to be necessary for the development of seizures (Huo, et al., 2006, Rice and DeLorenzo, 1998). Additionally, NMDAR phosphorylation increased directly after status epilepticus (Huo, et al., 2006, Moussa, et al., 2001, Niimura, et al., 2005). Yet, it remains to be established whether NMDAR phosphorylation changes after amygdala kindling and spontaneous seizures. Therefore we evaluated phosphorylation of the NMDAR in the amygdala kindling model and the self-sustained limbic status epilepticus model (SSLSE) (Lothman, et al., 1989). Furthermore, we evaluated the cellular and subcellular localization of the NMDAR in the latter model, because receptor localization is another important determinant of receptor functioning (Hardingham, et al., 2002, Papadia and Hardingham, 2007).

Vagus nerve stimulation

Many seizures can be treated by anticonvulsant pharmacotherapy. However, one third of all epilepsy patients do not respond to antiepileptic drugs or they have to discontinue pharmacotherapy because of severe side effects (Kwan and Brodie, 2000, Sander, 1993). Aside from having deleterious effects on health, persistent epileptic seizures can cause psychosocial, behavioural, and cognitive impairment and impose large restrictions on patients and their relatives.

Vagus nerve stimulation (VNS) is a surgical treatment for the large group of patients suffering from medically refractory epilepsy who are not eligible for resective surgery or for whom this surgery had failed. It consists of chronic intermittent electrical stimulation of the left vagus nerve, delivered by a programmable pulse generator. This device is subcutaneously implanted in the chest wall together with a battery pack and subsequently connected to a bipolar electrode that is wrapped around the vagus nerve in the neck (Figure 1.1). VNS can reduce seizure frequency and seizure severity. In addition, positive effects have been reported on mood, cognition, and behaviour. Moreover, contrary to many antiepileptic drugs, VNS has a favourable side effect profile. Side effects are mild and mainly restricted to the time the stimulator is delivering a pulse.

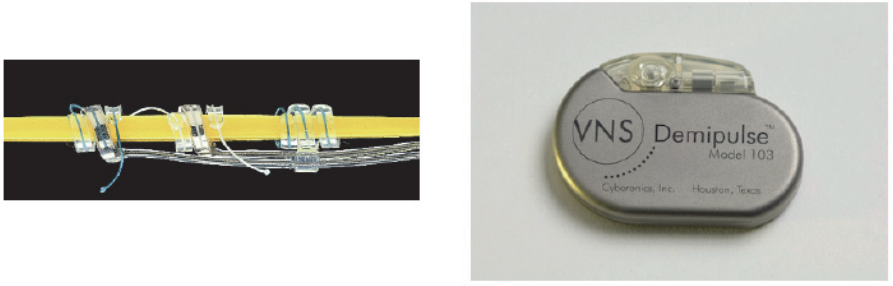


Figure 1.1 Vagus nerve stimulator.
Left: Bipolar electrode. Right: Pulse generator (Courtesy of Cyberonics Inc.)

Unfortunately, not all patients respond to VNS: 23-31% of VNS treated patients experience more than 50% seizure frequency reduction versus 13-16% of placebo treated patients (Handforth, et al., 1998, The Vagus Nerve Stimulation Study Group, 1995). It is unclear how VNS works and why it only works in a subgroup of patients. In this respect, one of the physiological functions of the vagus nerve might be relevant. It is known that the vagus nerve is involved in the so-called cholinergic anti-inflammatory reflex (Pavlov and Tracey, 2005). In this reflex, afferent vagus nerve fibres signal the brain that inflammation is occurring. Thereupon, the efferent fibres of the vagus nerve reduce the production of pro-inflammatory cytokines. In this way the vagus nerve confines inflammation and thereby protects the organism from the detrimental effects of pro-inflammatory cytokines. Possibly, VNS reduces inflammation in epilepsy as well, which might explain part of its anticonvulsive effects. Therefore, we aimed to evaluate whether VNS exerts an anti-inflammatory effect in patients suffering from medically refractory epilepsy.

Aims and outline of this thesis

The aim of this thesis is to further elucidate the role of inflammatory processes in epilepsy and VNS. In **chapter two**, we provide an extensive overview of the studies that have evaluated cytokine levels in patients with epilepsy. Cytokine levels in brain tissue of patients suffering from medically refractory epilepsy are evaluated in **chapter three**. The results of two preclinical studies are presented in chapter four and five. In **chapter four** we characterize inflammatory processes in the amygdala-kindling model, while in **chapter five** we describe NMDAR changes in the SSLSE model. Finally we focus on VNS in chapters six to eight. In **chapter six** we evaluate the efficacy of VNS in children with treatment resistant epilepsy and in **chapter seven** we explore whether these effects are related to changes in inflammation. Future directions for preclinical research concerning VNS are discussed in **chapter eight**. In **chapter nine** a summary of the findings described in this thesis is given and the implications of the findings are discussed.

References

Balosso, S., Maroso, M., Sanchez-Alavez, M., Ravizza, T., Frasca, A., Bartfai, T., and Vezzani, A., 2008. A novel non-transcriptional pathway mediates the proconvulsive effects of interleukin-1beta. Brain 131**,** 3256-3265.

Berg, A. T., Berkovic, S. F., Brodie, M. J., Buchhalter, J., Cross, J. H., van Emde Boas, W., Engel, J., French, J., Glauser, T. A., Mathern, G. W., Moshe, S. L., Nordli, D., Plouin, P., and Scheffer, I. E., 2010. Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. Epilepsia 51**,** 676-685.

Bien, C. G., Urbach, H., Schramm, J., Soeder, B. M., Becker, A. J., Voltz, R., Vincent, A., and Elger, C. E., 2007. Limbic encephalitis as a precipitating event in adult-onset temporal lobe epilepsy. Neurology 69**,** 1236-1244.

Billiau, A. D., Wouters, C. H., and Lagae, L. G., 2005. Epilepsy and the immune system: is there a link? Eur J Paediatr Neurol 9**,** 29-42.

Dalmau, J., Gleichman, A. J., Hughes, E. G., Rossi, J. E., Peng, X., Lai, M., Dessain, S. K., Rosenfeld, M. R., Balice-Gordon, R., and Lynch, D. R., 2008. Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. Lancet Neurol 7**,** 1091-1098.

de Boer, H. M., 2010. Epilepsy stigma: moving from a global problem to global solutions. Seizure 19**,** 630-636.

Eadie, M., 1995. Epilepsy-from the Sakikku to hughlings Jackson. J Clin Neurosci 2**,** 156-162.

Fisher, R. S., van Emde Boas, W., Blume, W., Elger, C., Genton, P., Lee, P., and Engel, J., Jr., 2005. Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). Epilepsia 46**,** 470-472.

Handforth, A., DeGiorgio, C. M., Schachter, S. C., Uthman, B. M., Naritoku, D. K., Tecoma, E. S., Henry, T. R., Collins, S. D., Vaughn, B. V., Gilmartin, R. C., Labar, D. R., Morris, G. L., 3rd, Salinsky, M. C., Osorio, I., Ristanovic, R. K., Labiner, D. M., Jones, J. C., Murphy, J. V., Ney, G. C., and Wheless, J. W., 1998. Vagus nerve stimulation therapy for partial-onset seizures: a randomized active-control trial. Neurology 51**,** 48-55.

Hardingham, G. E., Fukunaga, Y., and Bading, H., 2002. Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5**,** 405-414.

Huo, J. Z., Dykstra, C. M., and Gurd, J. W., 2006. Increase in tyrosine phosphorylation of the NMDA receptor following the induction of status epilepticus. Neurosci Lett 401**,** 266-270.

Kwan, P., and Brodie, M. J., 2000. Early identification of refractory epilepsy. N Engl J Med 342**,** 314-319.

Lothman, E. W., Bertram, E. H., Bekenstein, J. W., and Perlin, J. B., 1989. Self-sustaining limbic status epilepticus induced by 'continuous' hippocampal stimulation: electrographic and behavioral characteristics. Epilepsy Res 3**,** 107-119.

Magiorkinis, E., Sidiropoulou, K., and Diamantis, A., 2010. Hallmarks in the history of epilepsy: epilepsy in antiquity. Epilepsy Behav 17**,** 103-108.

Morimoto, K., Fahnestock, M., and Racine, R. J., 2004. Kindling and status epilepticus models of epilepsy: rewiring the brain. Prog Neurobiol 73**,** 1-60.

Moussa, R. C., Ikeda-Douglas, C. J., Thakur, V., Milgram, N. W., and Gurd, J. W., 2001. Seizure activity results in increased tyrosine phosphorylation of the N-methyl-D-aspartate receptor in the hippocampus. Brain Res Mol Brain Res 95**,** 36-47.

Niimura, M., Moussa, R., Bissoon, N., Ikeda-Douglas, C., Milgram, N. W., and Gurd, J. W., 2005. Changes in phosphorylation of the NMDA receptor in the rat hippocampus induced by status epilepticus. J Neurochem 92**,** 1377-1385.

Ozkara, C., and Vigevano, F., 2011. Immuno- and antiinflammatory therapies in epileptic disorders. Epilepsia 52 Suppl 3**,** 45-51.

Papadia, S., and Hardingham, G. E., 2007. The dichotomy of NMDA receptor signaling. Neuroscientist 13**,** 572-579.

Pavlov, V. A., and Tracey, K. J., 2005. The cholinergic anti-inflammatory pathway. Brain Behav Immun 19**,** 493-499.

Rice, A., and DeLorenzo, R., 1998. NMDA receptor activation during status epilepticus is required for the development of epilepsy. Brain Research 782**,** 240-247.

Sander, J. W., 1993. Some aspects of prognosis in the epilepsies: a review. Epilepsia 34**,** 1007-1016.

Sander, J. W., 2003. The epidemiology of epilepsy revisited. Curr Opin Neurol 16**,** 165-170.

The Vagus Nerve Stimulation Study Group, 1995. A randomized controlled trial of chronic vagus nerve stimulation for treatment of medically intractable seizures. Neurology 45**,** 224-230.

Tuunanen, J., and Pitkanen, A., 2000. Do seizures cause neuronal damage in rat amygdala kindling? Epilepsy Res 39**,** 171-176.

Vezzani, A., Conti, M., De Luigi, A., Ravizza, T., Moneta, D., Marchesi, F., and De Simoni, M. G., 1999. Interleukin-1beta immunoreactivity and microglia are enhanced in the rat hippocampus by focal kainate application: functional evidence for enhancement of electrographic seizures. J Neurosci 19**,** 5054-5065.

Vincent, A., Irani, S. R., and Lang, B., 2010. The growing recognition of immunotherapy-responsive seizure disorders with autoantibodies to specific neuronal proteins. Curr Opin Neurol 23**,** 144-150.

Viviani, B., Bartesaghi, S., Gardoni, F., Vezzani, A., Behrens, M. M., Bartfai, T., Binaglia, M., Corsini, E., Di Luca, M., Galli, C. L., and Marinovich, M., 2003. Interleukin-1beta enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. J Neurosci 23**,** 8692-8700.

Chapter 2

Cytokines in epilepsy: a critical review of the human literature

M. Aalbers, K. Rijkers, S. Klinkenberg, M. Majoie, M. de Baets, G. Hoogland, J. Vles

Submitted

Abstract

In recent years the role of cytokines in the pathophysiology of epilepsy has received increasing attention. The present review is a critical appraisal of all clinical studies that assessed cytokine levels in epilepsy syndromes without a prototypical inflammatory aetiology such as temporal lobe epilepsy. Most of these studies analyzed a limited number of pro-inflammatory cytokines. They demonstrated increased IL-6 levels and increased brain IL-1β levels, whereas TNF-α analyses were inconclusive. The relation between expression patterns and clinical parameters suggests that cytokine changes do not merely result from seizure activity. However, the functional interplay of different cytokines and their role in epileptogenesis remains elusive. The functional consequences, clinical implications, and future directions for research are discussed.

Introduction

Epilepsy is a chronic disorder characterized by the occurrence of recurrent spontaneous seizures. It is one of the most common neurological disorders affecting up to 1% of the world's population (Sander, 2003). Approximately one third of all patients are resistant to current drug therapy. Elucidation of the pathophysiological mechanisms of epilepsy could aid development of new antiepileptic treatments and thereby decrease therapy resistance.

Over the past years the role of inflammation has received increasing attention in the pathophysiology of epilepsy (Vezzani and Granata, 2005). Of special interest are cytokines that link the immune system to the nervous system. This review summarizes the clinical studies that determined cytokine levels in blood, cerebrospinal fluid (CSF), and brain tissue of patients suffering from epilepsy syndromes without a specific underlying inflammatory or autoimmune aetiology. Based on these studies, we describe new directions in clinical research. We searched Pubmed and The Cochrane Library for articles published until April 2012 using the following key words: 'epilepsy AND cytokines', 'epilepsy AND inflammation', 'seizures AND cytokines' and 'seizures AND inflammation'. We included articles identified from these searches and relevant references cited in the articles. Only original studies that were written in English and regarded human data were included. Finally, 33 papers remained for this review. Sixteen studies evaluated cytokine levels in brain tissue, seven in CSF, and fourteen in blood (Figure 2.1). Before discussing the relation between cytokines and epilepsy and the influence of antiepileptic treatment on cytokines, we first describe how cytokines may contribute to seizures, their origin in epilepsy, and how they can be assessed.

Pro- and anticonvulsive effects of cytokines

Cytokines are small proteins that function as signalling molecules in intercellular communication. Many cytokines have both pro- and anti-inflammatory properties and their net physiological effect depends on various factors, including receptor expression, concentration, timing, and the combination in which they are secreted. Basal cytokine expression in the central nervous system (CNS) is low, but rapid upregulation takes place after various types of CNS insults (Hopkins and Rothwell, 1995). The expression is also modulated by several neurotransmitters and neuropeptides (Szelenyi, 2001). Cytokines in their turn can alter the excitability of neuronal cells and thereby contribute to seizure generation. For instance, preclinical studies showed that the pro-inflammatory cytokine *interleukin-1beta* (IL-1β) is proconvulsive (Vezzani, et al., 1999). This proconvulsant activity may result from the direct influence of IL-1 on network excitability by affecting neurotransmitter balance: it increases glutamate release and inhibits astrocytic glutamate uptake (Hu, et al., 2000, Kamikawa, et al., 1998). Furthermore, IL-1 directly enhances NMDA-receptor functioning

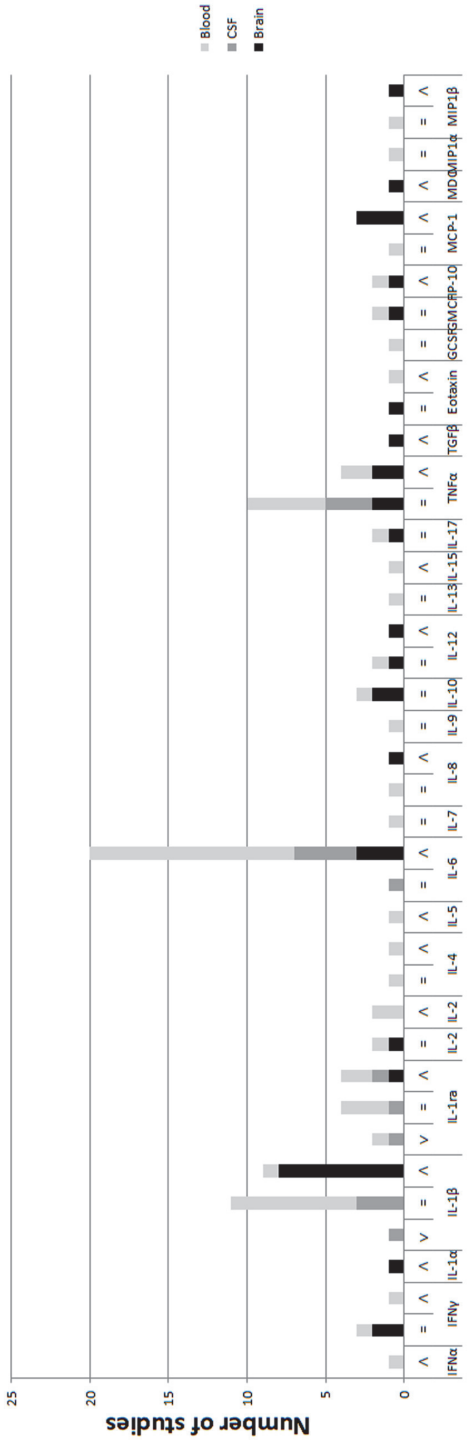


Figure 2.1 Number of studies that evaluated cytokine levels in human epilepsy. = = unaltered; V = decreased; Λ = increased.

by binding to interleukin-1 receptor type I (IL-1RI), thereby increasing neuronal excitability (Balosso, et al., 2008). Receptor binding also leads to activation of slower transcriptional pathways that result in structural and functional changes in glial and neuronal networks (Moynagh, et al., 1993). The IL-1-response is modulated by IL-1 receptor antagonist (IL-1ra), the natural antagonist of IL-1, which has anticonvulsive properties (Vezzani, et al., 2000).

Interleukin-6 (IL-6) mediates the acute phase reaction and fever, like IL-1. It has both pro- and anti-inflammatory properties. IL-6 binds to the IL-6 receptor (IL-6R) complex that consists of the ligand binding IL-6R and the signal-transducing glycoprotein130 (Gp130). In vitro, IL-6 can influence excitability by reducing inhibitory postsynaptic potentials (Garcia-Oscos, et al., 2011). Experimentally, IL-6 has ambiguous effects on seizure susceptibility (Fukuda, et al., 2007, Kalueff, et al., 2004).

One of the most studied members of the *Tumor Necrosis Factor* (TNF) superfamily is TNF-alpha (TNF-α), a pro-inflammatory cytokine that activates many different immune and non-immune cells and induces fever and sepsis. TNF-α functionally interacts with IL-1β and IL-6: they can induce each other's secretion and they synergize with one another to produce augmented effects. TNF-α modulates synaptic transmission by altering glutamate and GABA receptor expression and glutamate release (Bezzi, et al., 2001, Stellwagen, et al., 2005). Depending on the experimental model, TNF-α has been shown to have both proconvulsant and anticonvulsant properties (Balosso, et al., 2005, Yuhas, et al., 1999).

Interferons (IFN) are a subclass of cytokines named after their ability to interfere with viral replication. IFN-alpha (IFN-α) modulates specific cellular functions and IFN-gamma (IFN-γ) strongly regulates immune and inflammatory responses. IFNs directly induce epileptiform bursting by exciting hippocampal neurons and by lowering inhibitory postsynaptic potentials (Muller, et al., 1993).

Chemokines are a subfamily of chemotactic cytokines that guide migration of leukocytes. This leukocyte trafficking can contribute to epileptic seizures by inducing blood brain barrier (BBB) leakage. Furthermore, like other cytokines they can directly alter neuronal activity (for a review on chemokines in epilepsy see Fabene et al. (Fabene, et al., 2010)).

Origin of cytokines in epilepsy

Many studies have found elevated cytokine levels in brain tissue of epilepsy patients (Boer, et al., 2008, Boer, et al., 2007, Choi, et al., 2009, Feng, et al., 2011, Iyer, et al., 2010, Lu, et al., 2009, Maldonado, et al., 2003, Ravizza, et al., 2006, Ravizza, et al., 2008, Sheng, et al., 1994, Shu, et al., 2010, Varella, et al., 2011, Wu, et al., 2008). It is unclear whether production of these cytokines takes place peripherally or within the CNS. Many areas involved in seizure generation, including the hippocampus, are connected to the hypothalamic pituitary axis and can thereby affect the immune

system and cytokine production (Wrona, 2006). Leukocytes are the first possible peripheral source of cytokines in epilepsy. These cells can secrete cytokines and this secretion can be directly stimulated by neurotransmitters (Levite, 2008). Yet, epilepsy patients with high levels of circulating pro-inflammatory cytokines do not show an increased production of cytokines by peripheral blood mononuclear cells (PBMC), which suggests a different origin of cytokines in these patients (Hulkkonen, et al., 2004). As cytokines are also released from muscle during exercise (Pedersen and Hoffman-Goetz, 2000), seizure-related muscular activity could be a potential source of cytokines in epilepsy. However, this production is only relevant for specific seizure types, as not all seizures are accompanied by increased muscular activity. Moreover, cytokine levels are also increased in psychiatric patients with convulsions as a result of electroconvulsive therapy, even though these seizures are not accompanied by muscle contractions as these patients are anaesthetized (Lehtimaki, et al., 2008).
Peripherally produced cytokines may reach the CNS via activation of vagal afferents or directly at the circumventricular organs or the BBB. The latter site is particularly relevant in epilepsy, in which the BBB can be compromised (Boer, et al., 2008, Ravizza, et al., 2008). This origin of cytokines would be even more likely if cytokines were predominantly found in parenchyma surrounding blood vessels, a finding that was only mentioned in two studies (Ravizza, et al., 2008, Shu, et al., 2010). Higher concentrations in CSF than in plasma also argue in favour of a central origin (Peltola, et al., 1998, Peltola, et al., 2000). Moreover, cytokines and their receptors are expressed in the brain by neurons (Boer, et al., 2008, Ravizza, et al., 2006, Ravizza, et al., 2008), microglia (Boer, et al., 2008, Ravizza, et al., 2006, Ravizza, et al., 2008), and astrocytes (Boer, et al., 2008, Liu, et al., 2000, Lu, et al., 2009, Ravizza, et al., 2006, Ravizza, et al., 2008). In conclusion, cytokines produced in patients with epilepsy most likely originate from within the CNS.

Methodological aspects of assessing cytokine levels

A detailed overview of all studies included in this review is provided in Table 2.1.

Sample collection

Because cytokines in epilepsy most likely originate from the CNS, studies using resected brain tissue provide most direct information.
Sixteen such studies have been published (Boer, et al., 2008, Boer, et al., 2007, Choi, et al., 2009, Feng, et al., 2011, Fiala, et al., 2012, Iyer, et al., 2010, Liu, et al., 2000, Lu, et al., 2009, Maldonado, et al., 2003, Ravizza, et al., 2006, Ravizza, et al., 2008, Sheng, et al., 1994, Shu, et al., 2010, Varella, et al., 2011, Wu, et al., 2008, Yamamoto, et al., 2006). These studies only describe a specific subset of patients, because only patients with localized refractory epilepsy are eligible for resective surgery. They are further complicated by the lack of an ideal control, i.e. normal brain tissue. Instead, studies

used autopsy controls (Boer, et al., 2008, Boer, et al., 2007, Choi, et al., 2009, Iyer, et al., 2010, Lu, et al., 2009, Ravizza, et al., 2006, Ravizza, et al., 2008, Varella, et al., 2011, Yamamoto, et al., 2006) or resected brain tissue obtained during trauma (Feng, et al., 2011, Shu, et al., 2010, Wu, et al., 2008) and tumour surgery (Feng, et al., 2011, Liu, et al., 2000). Cytokine levels in these specimens might be influenced by post-mortem interval and inflammation associated with trauma and tumours, respectively. Other studies compared different epilepsy associated pathologies (Boer, et al., 2007, Iyer, et al., 2010, Maldonado, et al., 2003, Ravizza, et al., 2008) and lesional to perilesional tissue (Boer, et al., 2008, Maldonado, et al., 2003, Ravizza, et al., 2006).

CSF is probably the second best material to study CNS cytokine levels and has been used in seven studies (Haginoya, et al., 2009, Lehtimaki, et al., 2004, Lehtimaki, et al., 2010, Peltola, et al., 1998, Peltola, et al., 2000, Sinha, et al., 2008, Tekgul, et al., 2006). It is unknown how these cytokine levels relate to brain cytokine levels. They possibly differ because cytokines are rapidly eliminated from CSF (Banks, et al., 1994). Furthermore, local changes in CNS cytokine levels may be diluted and thus difficult to detect in CSF. Therefore, CSF analyses may miss out on important changes, because also small local changes can exert biologically significant effects (Dinarello, 1991). Finally, ethical reasons strongly limit CSF collection, which hampers longitudinal monitoring of CSF cytokine levels. Furthermore, control CSF is only available from patients in whom lumbar punction is clinically indicated for example to exclude neurological disease (Haginoya, et al., 2009, Lehtimaki, et al., 2004, Lehtimaki, et al., 2010, Peltola, et al., 1998, Peltola, et al., 2000) or for spinal anaesthesia for non-inflammatory disorders (Sinha, et al., 2008).

Finally, fourteen studies evaluated blood cytokine levels (Alapirtti, et al., 2009, Bauer, et al., 2009, Carmeli, et al., 2009, Hulkkonen, et al., 2004, Lehtimaki, et al., 2004, Lehtimaki, et al., 2007, Lehtimaki, et al., 2011, Liimatainen, et al., 2009, Liu, et al., 2001, Nowak, et al., 2011, Peltola, et al., 1998, Peltola, et al., 2000, Shiihara, et al., 2010, Sinha, et al., 2008). Similar to CSF, it is unclear how blood levels relate to CNS levels. The advantage of using blood is that it is relatively easy obtainable. It can be sampled repetitively enabling comparison within the same patients to evaluate seizure-associated changes (Alapirtti, et al., 2009, Bauer, et al., 2009, Lehtimaki, et al., 2007). Moreover, comparison to healthy controls is possible (Carmeli, et al., 2009, Lehtimaki, et al., 2011, Liimatainen, et al., 2009, Liu, et al., 2001, Nowak, et al., 2011, Shiihara, et al., 2010, Sinha, et al., 2008). Studies either used plasma (Alapirtti, et al., 2009, Hulkkonen, et al., 2004, Lehtimaki, et al., 2007, Peltola, et al., 1998, Peltola, et al., 2000) or serum (Bauer, et al., 2009, Carmeli, et al., 2009, Lehtimaki, et al., 2004, Lehtimaki, et al., 2011, Liimatainen, et al., 2009, Liu, et al., 2001, Nowak, et al., 2011, Shiihara, et al., 2010, Sinha, et al., 2008). This can affect cytokine measurement: in serum cellular elements can release cytokines during clotting, while anticoagulants in plasma samples can influence certain assays (Zhou, et al., 2010).

In short, CNS levels are best reflected by brain tissue, but are easier evaluated in CSF. Blood is the most suitable to study possible biomarkers due to its availability.

Table 2.1 Details of all studies that have determined cytokines changes in epilepsy depicted per study.

Study	Cytokine	Cytokine change	Epilepsy Syndrome/ Pathology (n)	Seizure	Refractory	Compared to (n)	Postictal interval (h)	Source	Method
Alapirtti 2009	IL-6	↑	TLE (11)	P/G	Yes	Inter vs postictal	3,6,12,24	Plasma	Luminex
	IL-1β, IL-1ra	= TLE/ eTLE	eTLE (9)						ELISA
Bauer 2009	IL-6	↑	TLE	P/G	Yes	Inter vs postictal	0.16,1,24	Serum	ELISA
	IL-1β, TNF-α	=							
Boer 2006	IL-1β	↑	HMEG (1)	G/SE	Yes	Autopsy tissue (3)	n.m.	Cortex	IHC
						HMEG (sporadic) (3)			
			HMEG (1)		Yes	Controlat cortex (2)			
Boer 2008	IL-1β, IL-1RI	↑	TSC (9)	P/G	Yes	Perilesional tissue (4)	n.m.	Lesion	IHC
			SGCT (1)	P/G		Autopsy tissue (5)			
Carmeli 2009	IL-6	↑	n.m. (19)	n.m.	n.m.	Matched control (10)	n.m.	Serum	RT-PCR WB
Choi 2009	IL-1β IL8 IL-12 MIP-1β	↑	FCD (6)	n.m.	Yes	Autopsy tissue (2)	n.m.	Cortex	ELISA
	IL-6 IP-10 MCP-1MDC	↑ Some patients	EM (5)						
	Eotaxin eotaxin-3 GM-CSF	=	TLE (1)						
	IFNγ IL-2 IL-10		Rasmussen (1)						
	MCP-4 TARC TNFa								
Feng, 2011	IL-1β, IL-6, TGF-β1	↑	TLE (30)	P/G	Yes	Trauma (8)/ tumor (2)	n.m.	Temporal lobe	Multi ELISA
	IFNγ, IL-10, IL-12, IL-17a, TNF-α	=							
Fiala 2012	RANTES	↑	TLE (13)	P	Yes	Autopsy tissue (3)	n.m.	Cortex/	IHC
	IL-1β, MIP-1α, MIP-1β, CXCR4, CCR5	Detectable				Trauma (1)/ tumor (1)		hippocampus	
Haginoya 2009	IL-1β IL-6 TNF-α	=	WS (31)	G	No treatment	Exclusion Disease	After spasm	CSF	ELISA
	IL-1ra	↓							
Hulkkonen 2004	IL-6	↑	TLE (8)	n.m.	Yes	Healthy control (200)	>48	Plasma	ELISA
	IL-1β	=	FLE (2)						
	IL-1ra	↓							
Iyer 2010	IL-1β, MCP-1	↑ Highest in FCD type IIB	FCD type IA (8)	P	Yes	Epilepsy nonFCD (4)	n.m.	Lesion	IHC
			FCD type IIB (9)			Autopsy tissue (6)			
Lehtimäki 2004	IL-6	↑ Highest in rGTCS	Symptomatic (8)	P/G	n.m.	Exclusion	n.m.	CSF	ELISA
	gp130	↑ Only serum sGTCS	Cryptogenic (14)			Disease (17)		Serum	
	sIL-6R	↓	Provoked (10)						

Study	Cytokine	Cytokine change	Epilepsy Syndrome/ Pathology (n)	Seizure	Refractory	Compared to (n)	Postictal interval (h)	Source	Method
Lehtimäki 2007	IL1ra, IL-6	↑	TLE (9)	P/G	No	Inter vs postictal (8)	1,2,12,24	Serum	ELISA
	IL-1β, sIL-6R, sGp130	=	FLE (2)						
			MFE (1)						
Lehtimäki 2010	IL-1ra	↑ Only after rTCS/pPS	Symptomatic (5)	P/G	n.m.	Exclusion	<24	CSF	ELISA
	IL-1β	↓ Only after rTCS/pPS	Cryptogenic (13)			Disease (16)			
			Provoked						
Lehtimäki 2011	IL-6	↑	Encephalopathy (30)	n.m.	Yes	Healthy control (63)	n.m.	Serum	ELISA
			Symptomatic (38)						
			Generalized idiopathic(5)						
Liimatainen 2009	IL-6	↑ Higher in TLE vs eTLE	TLE (63)	n.m.	Yes (86)	Healthy control (63)	>24	Serum	ELISA
	IL-1Ra	=	eTLE (28)		No (5)				
Liu 2000	IL-4Rα	Present	TLE (4)	n.m.	Yes	Glial tumour (4)	n.m.	Cortex	IHC/RT-PCR/SB
Liu 2001	IFNα, IL-2, TNF-α	↑ IL-2 highest in symptomatic	WS (23)	G	No treatment	Healthy control (15)	n.m.	Serum	ELISA
Lu 2009	TGFβR-I	↑	TLE (30)	P/G	Yes	Autopsy control (8)	n.m.	Cortex	IHC/ WB
Maldonado 2003	TNF-α	↑	TSC resection (10)	n.m.	Yes (n=10)	Perilesional (5)	n.m.	Lesion	IHC/ WB
			TSC autopsy (3)			FCD (3)			
						Non FCD TSC (10)			
						Autopsy control (4)			
Nowak 2011	IL-6	↑	Focal (75)	n.m.	No	Healthy control (36)	>72	Serum	ELISA
	IL-1β, TNF-α	=	Generalized (26)						
Peltola 1998	IL-6	↑ Only if interval <72h	Symptomatic epilepsy (4)	G	No treatment	Exclusion disease (22)	>10 -<72	CSF/serum	ELISA
	IL-1β, TNF-α	=					>2w		
Peltola 2000	IL-6	↑	n.a.	G	No treatment	Exclusion disease (18)	<24h	CSF/serum	ELISA
	IL-1β, IL-1ra, TNF-α	=							
Ravizza 2006	IL-1β IL-1ra IL-1RI IL-1RII	↑	FCD (9)	P/G	Yes	Perilesional (6)	>24	Lesion	IHC
			Ganglioma (9)			Autopsy tissue (6)			
			DNT (9)						

Study	Cytokine	Cytokine change	Epilepsy Syndrome/ Pathology (n)	Seizure	Refractory	Compared to (n)	Postictal interval (h)	Source	Method
Ravizza 2008	IL-1β	↑	TLE-HS (12) TLE-non HS (6)	P/G	Yes	Autopsy tissue (6)	>24	Hippocampus	IHC
Sheng 1994	IL-1α	↑	HS/ gliosis (5) Non-HS (3)	P	Yes	Autopsy tissue (8)	n.m.	Cortex/ Hippocampus	IHC
Shiihara 2010	IL-1ra, IL-5, IL-6, IL-15, eotaxin, IP-10 IL-1β IL-2 IL-4 IL-7 IL-8 IL-9 IL-10 IL-12 IL-13 IL-17 IFNγ TNF-α, GCSF GMCF MCP-1, MIP-1α, MIP-1β	↑ =	WS (70)	G	No treatment	Healthy control (26)	n.m.	Serum	Multiplex
Sinha 2008	IFN-γ, IL-1β, IL-2, IL4, IL-6, TNF-α	↑	Focal (47) Generalized (53)	P/G/SE	No	Healthy control (100) Spinal anesthesia	<24	CSF/Serum	ELISA
Shu 2010	IL-6, IL-6R, sGp130	↑ In FCD, TSC	FCD (11) TSC (20)	P/G	n.m.	Normal cortex Trauma patients (10)	n.m.	Lesion	RT-PCR/ WB IHC/ ELISA
Tekgul 2006	IL-6	↓	WS (10)	G	n.m.	Trauma+seizure (5) Infectious seizures (12)	1-6	CSF	ELISA
Varella 2011	IL-1β, TNF-α	↑ TNF only shown by ELISA	TLE (29)	n.m.	Yes	Autopsy tissue (10)	n.m.	Hippocampus	ELISA/ IHC
Wu 2008	MCP-1	↑	TLE (40)	P/G	Yes	Post-trauma surgical specimens (8)	n.m.	Cortex/ Hippocampus	IHC/ WB
Yamamoto 2006	TNFRI	↑	TLE (10)	n.m.	Yes	Autopsy tissue	n.m.	Hippocampus	WB

Interval is the time between last seizure and cytokine determination. CSF=cerebrospinal fluid; DNT=dysembryoplastic neuroepithelial tumors; ELISA=enzyme-linked immunosorbent assay; EM=encephalomalacia; eTLE=extratemporal lobe epilepsy; FCD=focal cortical dysplasia; FLE=frontal lobe epilepsy; G=generalized; h=hours; HMEG=hemimegalencephaly; HS=hippocampal sclerosis; IFN=interferon; IHC=immunohistochemistry; IL=interleukin; MFE=multifocal epilepsy; n.a.=not applicable; n.m.=not mentioned; P=partial; RIA=radio-immunoassay; RT-PCR=reverse transcription polymerase chain reaction; pPS=prolonged partial seizure; rGTCS=recurrent generalized tonic clonic seizure; rTCS=recurrent tonic-clonic seizures; SB=southern blot; SE=status epilepticus; SGTC=supependymal giant cell tumors; sGTCS=single generalized tonic-clonic seizure; TGFβR-I=type I transforming growth factor β receptor; TLE=temporal lobe epilepsy; TNF=tumor necrosis factor; TSC=tuberous sclerosis complex; w=weeks; WB=Western Blot; WS=West Syndrome; ↑ increase; ↓ decrease; = equal.

Cytokine analysis

In general analysis of cytokines is challenging due to their complex biology. First of all, many cytokines exhibit diurnal variations. Several studies controlled for this by acquiring samples at the same time point (Bauer, et al., 2009, Liimatainen, et al., 2009, Peltola, et al., 1998, Peltola, et al., 2000). Furthermore, concentration changes are difficult to detect, while cytokines usually act over short distances and short time spans. Finally, the low basal expression hinders detection of decreased levels. This is illustrated by the fact that only three studies observed decreased levels (Haginoya, et al., 2009, Hulkkonen, et al., 2004, Lehtimaki, et al., 2010).

Brain levels are mainly evaluated qualitatively by immunohistochemistry (Boer, et al., 2008, Boer, et al., 2007, Iyer, et al., 2010, Liu, et al., 2000, Lu, et al., 2009, Maldonado, et al., 2003, Ravizza, et al., 2006, Ravizza, et al., 2008, Sheng, et al., 1994, Shu, et al., 2010, Varella, et al., 2011, Wu, et al., 2008). This allows evaluation of regional and cellular expression, but provides little data on cytokine concentrations. Semi-quantitative analysis of protein, DNA, and m-RNA levels was performed using Western Blot (Carmeli, et al., 2009, Lu, et al., 2009, Maldonado, et al., 2003, Shu, et al., 2010, Wu, et al., 2008, Yamamoto, et al., 2006), Southern blot (Liu, et al., 2000), and RT-PCR (Carmeli, et al., 2009, Liu, et al., 2000, Shu, et al., 2010). A drawback of these latter two approaches is that DNA and mRNA levels do not necessarily reflect secreted protein levels.

Cytokine levels in CSF and blood were mostly studied by enzyme linked immunosorbance assay (ELISA) (Alapirtti, et al., 2009, Bauer, et al., 2009, Haginoya, et al., 2009, Hulkkonen, et al., 2004, Lehtimaki, et al., 2004, Lehtimaki, et al., 2007, Lehtimaki, et al., 2010, Lehtimaki, et al., 2011, Liimatainen, et al., 2009, Liu, et al., 2001, Nowak, et al., 2011, Peltola, et al., 1998, Peltola, et al., 2000, Sinha, et al., 2008, Tekgul, et al., 2006). Though ELISA is a well-validated technique that detects protein concentrations in the range of tenths of picograms per millilitre, it can only assess one cytokine at a time. This disadvantage is overcome by multiplex analysis, by which several cytokines can be determined simultaneously. This analysis therefore better captures the complexity of an inflammatory response, but so far just two studies applied this method in this way (Choi, et al., 2009, Shiihara, et al., 2010). As all these methods have their pros and cons, they should be regarded as complimentary approaches.

Cytokine expression after a seizure

There is a close temporal relation between changes in cytokine levels and the occurrence of seizures. For instance, IL-6 levels are higher in plasma and CSF in patients who had had recent epileptic seizures than in control subjects (Peltola, et al., 1998, Peltola, et al., 2000). These levels were equal when the seizures occurred more

than two weeks earlier (Peltola, et al., 1998). This increase of IL-6 already occurs immediately after a single seizure (Bauer, et al., 2009), reaching peak levels at six hours (Alapirtti, et al., 2009, Lehtimaki, et al., 2007). For other cytokines results were more conflicting. Sinha et al only evaluated the presence or absence of cytokines and demonstrated that IFN-γ, IL-1β, IL-2, IL-4, IL-6, and TNF-α were more often detectable in patients than in controls (Sinha, et al., 2008). However, others who determined postictal cytokine concentrations mostly found similar levels of IL-1β and TNF-α in patients and controls (Haginoya, et al., 2009, Peltola, et al., 1998, Peltola, et al., 2000). IL-1ra levels were decreased (Haginoya, et al., 2009), increased (Lehtimaki, et al., 2007, Lehtimaki, et al., 2010), or unaltered (Peltola, et al., 2000). These inconsistent observations might result from differences in timing of sample collection and cytokine assessment methods.
In addition to a temporal relationship, cytokine expression also appears to be related to seizure severity. A decrease of CSF IL-1β and an increase in IL-1ra was observed in patients that experienced recurrent tonic-clonic or prolonged partial seizures, but not in patients with a single tonic-clonic seizure (Lehtimaki, et al., 2010). Similarly, IL-6 levels were highest in patients with recurrent generalized tonic clonic seizures (Lehtimaki, et al., 2004).

Cytokine expression in epilepsy

In addition to acute seizure induced changes, cytokine levels are also altered in the chronic epileptic state during the interictal period. Serum levels of IL-6 can be increased up to 72 hours after a seizure (Hulkkonen, et al., 2004, Liimatainen, et al., 2009, Nowak, et al., 2011), yet blood levels of IL-1β and TNF-α were unaltered (Hulkkonen, et al., 2004, Nowak, et al., 2011). Levels of IL-1ra were either unaltered (Liimatainen, et al., 2009) or decreased (Hulkkonen, et al., 2004).
Brain levels of IL-1β, IL-6, and MCP-1 were unequivocally increased, both in TLE (Choi, et al., 2009, Feng, et al., 2011, Ravizza, et al., 2008, Sheng, et al., 1994, Shu, et al., 2010, Varella, et al., 2011, Wu, et al., 2008), and various epilepsy-associated malformations (Boer, et al., 2008, Boer, et al., 2007, Choi, et al., 2009, Iyer, et al., 2010, Ravizza, et al., 2006, Shu, et al., 2010). The expression of IL-1RI (Boer, et al., 2008, Ravizza, et al., 2006, Ravizza, et al., 2008) and IL-6R (Shu, et al., 2010) was increased as well. Studies regarding TNF-α obtained more variable results: one demonstrated an increase (Maldonado, et al., 2003), one unaltered levels (Choi, et al., 2009), and one an increase but only by ELISA and not by immunohistochemistry (Varella, et al., 2011). Moreover, TNF-RI (Yamamoto, et al., 2006) and IL-4 receptor (Liu, et al., 2000) were expressed in brain tissue of TLE patients. It is unclear whether this expression of IL-4 receptor is specific for epilepsy patients because no control samples were analysed (Liu, et al., 2000). Finally, Choi et all also found increased levels of several chemokines, such as IL-8, macrophage inflammatory protein-1beta, and macrophage derived

chemokine, and IFN-γ induced protein-10, in cortical specimens of some but not of all patients (Choi, et al., 2009).
Several reasons may explain these chronically altered cytokine levels. First of all, the cumulative effect of recurrent seizures might give rise to chronically altered cytokine levels. Indeed, the number of IL-1β and IL-1RI positive cells correlated with seizure frequency (Ravizza, et al., 2006) and patients with low seizure frequency (less than once a month) had similar IL-6 levels as controls (Lehtimaki, et al., 2011).
However, seizure activity itself appears to be insufficient to alter cytokine expression: cytokines are only found in epileptogenic, lesional cortex, and not in peri-lesional tissue, even though this peri-lesional cortex is exposed to seizure activity as well (Boer, et al., 2008, Maldonado, et al., 2003, Ravizza, et al., 2006). The underlying pathology might also be a determinant of cytokine expression. For instance, IL-1α positive cells in TLE are located adjacent to β-amyloid precursor protein positive neurons (Sheng, et al., 1994). Since both IL-1 and amyloid precursor protein are associated with neuronal injury, the IL-1α expression may result from neuronal injury that may be secondary to seizure activity (Panegyres, 1998). Ravizza et al showed that IL-1β and IL-1RI are only expressed in resected hippocampi from patients with hippocampal sclerosis, and not in patients with extrahippocampal epileptogenesis (Ravizza, et al., 2008). As both groups were comparable in terms of seizure type and frequency, this suggests that the expression is associated with this specific hippocampal pathology, i.e. neuronal cell loss and astrogliosis. Similarly, hippocampal expression of nuclear factor kappa B (NFκB), a transcription factor involved in inflammatory processes, was only enhanced in patients with hippocampal sclerosis (Crespel, et al., 2002). On the other hand, IL-1β levels were increased in cortical samples from patients with different types of epilepsy (Choi, et al., 2009) and in various epilepsy-associated malformations such as tuberous sclerosis (Boer, et al., 2008, Maldonado, et al., 2003), focal cortical dysplasia (Iyer, et al., 2010, Ravizza, et al., 2006), glioneuronal tumors (Ravizza, et al., 2006), and hemimegalencephaly (Boer, et al., 2007).
In sum, the chronic epileptic state is associated with altered cytokine levels that are related to the occurrence of repetitive seizures, but also to other factors such as the epilepsy associated pathology.

Effects of antiepileptic treatments on cytokine levels

Antiepileptic drugs

The interpretation of the above-mentioned findings is complicated by the effects of antiepileptic drugs on cytokine levels (see Table 2.2).
In vitro, production of IFNγ and IL-2 by mononuclear cells of healthy volunteers is reduced by diazepam (Wei, et al., 2010) and high doses of phenobarbital (Yang, et al., 1992), respectively. Moreover, valproate (VPA), but not carbamazepine (CBZ), inhibits NFκB activation and TNF-α and IL-6 production in human leukaemia cell lines

(Ichiyama, et al., 2000). On the contrary, in blood of epilepsy patients, CBZ administration directly stimulated IL-6 production (Mathieu, et al., 2011) and production of IL-1α, IL-1β, and IL-6 by PBMC was higher in patients using VPA or CBZ than in healthy controls (Pacifici, et al., 1995, Verrotti, et al., 2001).
In vivo studies have revealed that VPA increases plasma levels of IL-6 in healthy volunteers (Shiah, et al., 2005) and of MCP-1 in epilepsy patients (Verrotti, et al., 2001). Also, IL-5 (Makis, et al., 2005), IL-1β, and TNF-α (Bauer, et al., 2009) serum levels are higher in VPA treated epilepsy patients than in patients who are treated with other AEDs. Furthermore CBZ also increased MCP-1 serum levels (Verrotti, et al., 2001). Polytherapy is a common problem in these *in vivo* studies that hampers a clear interpretation of the contribution of individual AEDs to the observed cytokine changes. For instance, IL-5 and IL-10 serum levels are higher in patients receiving CBZ plus VPA, compared to patients who receive CBZ monotherapy (Mathieu, et al., 2011).
The modulating effects of AEDs on cytokine levels have clinical implications as well because they are linked to treatment response: For instance, IL-5 levels are higher in children with idiopathic epilepsy that responded to VPA treatment than in non-responders (Makis, et al., 2005). Moreover, relieve of West syndrome symptoms is associated with increased serum IL-ra (Yamanaka, et al., 2010), and decreased IL-1β, IL-12, and MIP-1β (Shiihara, et al., 2010).

Vagus nerve stimulation

Vagus nerve stimulation (VNS) is an adjunctive therapy for medically refractory epilepsy. Peripheral stimulation of the vagus nerve exerts neuroimmunological effects by activation of the cholinergic anti-inflammatory pathway. This pathway inhibits production of pro-inflammatory cytokines and thereby confines inflammation (Tracey, 2007). Activation of anti-inflammatory pathways by VNS may therefore partly explain its anticonvulsive effects.
Three studies have been published on VNS effects on cytokine levels in epilepsy patients. Two studies found that VNS did not alter IL-6, IL-10, and TNF-α (Barone, et al., 2007, Majoie, et al., 2011). One study reported a VNS-related anti-inflammatory effect (De Herdt, et al., 2009). This study showed that lipopolysaccharide-induced IL-8 production by mononuclear cells was diminished in cells that were obtained from patients who had received VNS for 6 months. Furthermore, patients suffering from refractory depression and who were treated for three months with VNS displayed increased levels of IL-6, TNF-α, and TGF-β (Corcoran, et al., 2005). These studies were mainly explorative, hampering evaluation of cytokine profiles in relation to treatment outcome and clinical characteristics.

Table 2.2 Effect of anti-epileptic drugs on cytokine levels and production.

Study	Cytokine	Cytokine change	AEDs	In/ ex vivo	Material	Source material (n)	Compared with
Ichiyama 2010	IL-6, TNF-α	↓	VPA	Ex	THP-1	n.a.	Untreated
	L-6, TNF-α	=	CBZ, DZP, PB, PHT				
Makis 2005	IL-5	↑	VPA	In	Serum	Epilepsy patients (68)	Healthy controls
Mathieu 2011	IL-5, IL-10	↑ VPA +CBZ	CBZ, CBZ+VPA, CBZ+LTG	In	Blood	Epilepsy patients (24)	Between AEDs
	IL-6	↑	CBZ	Ex			Baseline
	IL-2, IL-5, IL-10, IFN-γ	=					
Pacifici 1995	IL-1α, IL-1β, IL-6	↑	VPA, CBZ, PB	Ex	PBMC	Epilepsy patients (76)	Healthy controls
	IL-2	↑	CBZ				
Shihah 2005	IL-6	↑	VPA	In	Plasma	Healthy controls (10)	Baseline
Verrotti 2001	IL-1α, IL-1β, IL-2, IL-6	↑	CBZ	Ex	PBMC	Epilepsy patients (40)	Baseline/
	IL-1α, IL-1β, IL-6	↑	VPA				Healthy controls
	MCP-1	↑	CBZ, VPA	In	Plasma		
Wei 2010	IFN-γ	↓	DZP	Ex	PBMC	Healthy controls (15)	Untreated
Yang 1992	IL-2	↓	PB	Ex	Lymfocyt	n.m.	Untreated
	IL-1β, TNF-α	=					

AED=anti-epileptic drug; CBZ=carbamazepine; DZP=diazepam; IFN=interferon; IL=interleukin; LTG=lamotrigine; MCP=monocyte chemotactic protein; n.a.=not applicable; n.m.=not mentioned; PB=phenobarbital; PBMC=peripheral blood mononuclear cell; PHT=phenytoin; THP-1=human acute monocytic leukemia cell line; TNF=tumor necrosis factor; VPA=valproate; ↑ increase; ↓ decrease; = equal.

Ketogenic diet

The ketogenic diet is a high fat, low carbohydrate diet that is used in therapy resistant epilepsy. Its anticonvulsive activity may be partly mediated through the induction of anti-inflammatory processes. For instance, ketogenic metabolism reduces production of free oxygen species and thereby contributes to a decreased inflammatory state (Kim do, et al., 2007). In addition, the high fatty acid load may activate the peroxisome proliferator-activated receptor alpha that blocks pro-inflammatory transcription factors (Cullingford, 2008). The ketogenic diet was shown to influence inflammation in preclinical studies (Jeong, et al., 2011), but there are no data on the effects in epilepsy patients.

Clinical implications and future directions

The above-described studies have demonstrated seizure, epilepsy, and pathology associated increases in cytokine levels. As cytokines influence neuronal excitability, increased cytokine levels can facilitate the occurrence of new seizures (Figure 2.2), thereby constituting a positive feedback loop. However, due to the cross-sectional character of most studies, it is unclear to what extent these cytokine changes contribute to the initial transition of a healthy brain to a seizure-prone brain. As this process of epileptogenesis has already taken place when patients are diagnosed with epilepsy, it can only be studied in animal models. Therefore, studies aiming to elucidate inflammatory processes in epilepsy should go hand in hand with preclinical studies.

Furthermore, until now many studies have evaluated a limited number of cytokines only. This hampers understanding of the true situation: a decrease of one cytokine might actually reflect an increase of another cytokine. Moreover, the combination of different cytokines in concert determines their final effect. For example, the effects of IL-1β are confined by a 100-1000 fold excess of IL-1ra (Dinarello, 1996). A small increase in IL-1ra levels can therefore still fail to attenuate the effects IL-1β. Thus, future clinical studies should focus on a large spectrum of cytokines, which should also include cytokines that have received little attention so far. It may be equally important to assess expression levels of cytokine receptors, because receptor binding of cytokines can lead to underestimation of cytokine levels and because receptor expression can determine the outcome of an inflammatory reaction.

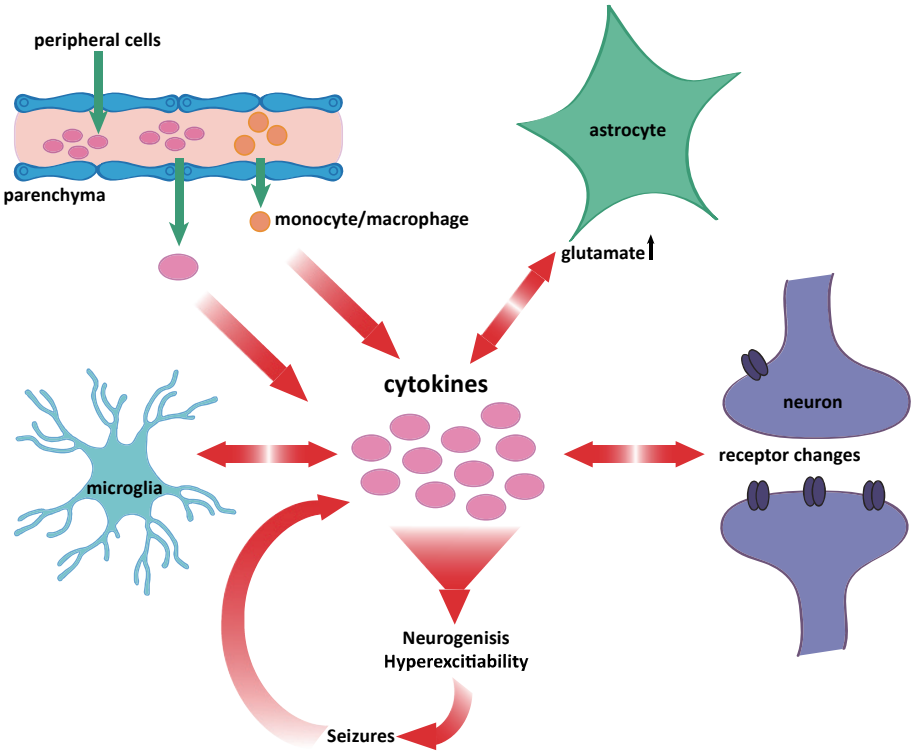


Figure 2.2 Involvement of cytokines in epilepsy.
Cytokines can be released by various sources. They exert slow transcriptional effects, but also directly influence neurotransmitter levels. Moreover, they quickly enhance receptor functioning and influence receptor composition. This contributes to increased network excitability and the generation of seizures.
Seizures and seizure associated pathological changes in their turn further enhance the production of cytokines, thereby establishing a reinforcing feedback loop.

Several studies demonstrated the importance of the underlying neuropathology and aetiology. However, in most studies patient numbers were too small to correlate clinical characteristics with cytokine changes. Future research should therefore use well-defined and large patient groups. Furthermore, research studying brain inflammation should extend beyond the evaluation of surgical specimens, in order to shed light on cytokine changes in the majority of patients, which are not eligible for epilepsy surgery. In this respect, recent developments in neuroimaging hold a promise for *in vivo* assessment of neuroinflammation. For example, iron oxide contrast-enhanced MRI and several nuclear imaging approaches have already visualized inflammation in multiple sclerosis and stroke (Stoll and Bendszus, 2009, Wunder, et al., 2009).

These future studies may result in the development of epilepsy-specific immunomodulatory treatments. General anti-inflammatory therapies such as intravenous immunoglobulins, corticosteroids, non-steroidal anti-inflammatory drugs, and adrenocorticotropic hormone, are already used in some epilepsy syndromes. Identification of key players in cytokine networks could aid the development of specific anti-inflammatory treatments. Such treatments are already successfully applied in other disorders such as psoriasis and rheumatoid arthritis. They seem a promising novel approach in epilepsy as well, as a preliminary study in four children suffering from refractory seizures reported a decreased seizure activity after treatment with Anakinra (IL-1ra) (Rahimian and Jyonouchi, 2011).

Conclusion

Although the involvement of cytokines in epilepsy has been clearly demonstrated, changes in certain cytokines and their interplay remain elusive. Most studies focused on pro-inflammatory cytokines, unequivocally showing increased IL-6 and increased brain IL-1β levels, whereas TNF-α analyses were inconclusive.
Expression patterns and correlation with clinical parameters suggest that cytokine changes do not merely result from seizure activity. Future studies should apply techniques that permit the analysis of a broad array of cytokines as well as their receptors. This could aid the development of inflammation modulating therapies that are specific for epilepsy.

References

Alapirtti, T., Rinta, S., Hulkkonen, J., Makinen, R., Keranen, T., and Peltola, J., 2009. Interleukin-6, interleukin-1 receptor antagonist and interleukin-1beta production in patients with focal epilepsy: A video-EEG study. J Neurol Sci 280**,** 94-97.

Balosso, S., Maroso, M., Sanchez-Alavez, M., Ravizza, T., Frasca, A., Bartfai, T., and Vezzani, A., 2008. A novel non-transcriptional pathway mediates the proconvulsive effects of interleukin-1beta. Brain 131**,** 3256-3265.

Balosso, S., Ravizza, T., Perego, C., Peschon, J., Campbell, I. L., De Simoni, M. G., and Vezzani, A., 2005. Tumor necrosis factor-alpha inhibits seizures in mice via p75 receptors. Ann Neurol 57**,** 804-812.

Banks, W. A., Kastin, A. J., and Gutierrez, E. G., 1994. Penetration of interleukin-6 across the murine blood-brain barrier. Neurosci Lett 179**,** 53-56.

Barone, L., Colicchio, G., Policicchio, D., Di Clemente, F., Di Monaco, A., Meglio, M., Lanza, G. A., and Crea, F., 2007. Effect of vagal nerve stimulation on systemic inflammation and cardiac autonomic function in patients with refractory epilepsy. Neuroimmunomodulation 14**,** 331-336.

Bauer, S., Cepok, S., Todorova-Rudolph, A., Nowak, M., Koller, M., Lorenz, R., Oertel, W. H., Rosenow, F., Hemmer, B., and Hamer, H. M., 2009. Etiology and site of temporal lobe epilepsy influence postictal cytokine release. Epilepsy Res 86**,** 82-88.

Bezzi, P., Domercq, M., Brambilla, L., Galli, R., Schols, D., De Clercq, E., Vescovi, A., Bagetta, G., Kollias, G., Meldolesi, J., and Volterra, A., 2001. CXCR4-activated astrocyte glutamate release via TNFalpha: amplification by microglia triggers neurotoxicity. Nat Neurosci 4**,** 702-710.

Boer, K., Jansen, F., Nellist, M., Redeker, S., van den Ouweland, A. M., Spliet, W. G., van Nieuwenhuizen, O., Troost, D., Crino, P. B., and Aronica, E., 2008. Inflammatory processes in cortical tubers and subependymal giant cell tumors of tuberous sclerosis complex. Epilepsy Res 78**,** 7-21.

Boer, K., Troost, D., Spliet, W. G., Redeker, S., Crino, P. B., and Aronica, E., 2007. A neuropathological study of two autopsy cases of syndromic hemimegalencephaly. Neuropathol Appl Neurobiol 33**,** 455-470.

Carmeli, E., Beiker, R., and Morad, M., 2009. Nitric oxide and interlukin-6 levels in intellectual disability adults with epilepsy. Res Dev Disabil 30**,** 567-571.

Choi, J., Nordli, D. R., Jr., Alden, T. D., DiPatri, A., Jr., Laux, L., Kelley, K., Rosenow, J., Schuele, S. U., Rajaram, V., and Koh, S., 2009. Cellular injury and neuroinflammation in children with chronic intractable epilepsy. J Neuroinflammation 6**,** 38.

Corcoran, C., Connor, T. J., O'Keane, V., and Garland, M. R., 2005. The effects of vagus nerve stimulation on pro- and anti-inflammatory cytokines in humans: a preliminary report. Neuroimmunomodulation 12**,** 307-309.

Crespel, A., Coubes, P., Rousset, M. C., Brana, C., Rougier, A., Rondouin, G., Bockaert, J., Baldy-Moulinier, M., and Lerner-Natoli, M., 2002. Inflammatory reactions in human medial temporal lobe epilepsy with hippocampal sclerosis. Brain Res 952**,** 159-169.

Cullingford, T., 2008. Peroxisome proliferator-activated receptor alpha and the ketogenic diet. Epilepsia 49 Suppl 8**,** 70-72.

De Herdt, V., Bogaert, S., Bracke, K. R., Raedt, R., De Vos, M., Vonck, K., and Boon, P., 2009. Effects of vagus nerve stimulation on pro- and anti-inflammatory cytokine induction in patients with refractory epilepsy. J Neuroimmunol 214**,** 104-108.

Dinarello, C. A., 1991. Interleukin-1 and interleukin-1 antagonism. Blood 77**,** 1627-1652.

Dinarello, C. A., 1996. Biologic basis for interleukin-1 in disease. Blood 87**,** 2095-2147.

Fabene, P. F., Bramanti, P., and Constantin, G., 2010. The emerging role for chemokines in epilepsy. J Neuroimmunol 224, 22-27.

Feng, Z. H., Hao, J., Ye, L., Dayao, C., Yan, N., Yan, Y., Chu, L., and Shi, F. D., 2011. Overexpression of mu-calpain in the anterior temporal neocortex of patients with intractable epilepsy correlates with clinicopathological characteristics. Seizure 20, 395-401.

Fiala, M., Avagyan, H., Merino, J. J., Bernas, M., Valdivia, J., Espinosa-Jeffrey, A., Witte, M., and Weinand, M., 2012. Chemotactic and mitogenic stimuli of neuronal apoptosis in patients with medically intractable temporal lobe epilepsy. Pathophysiology.

Fukuda, M., Morimoto, T., Suzuki, Y., Shinonaga, C., and Ishida, Y., 2007. Interleukin-6 attenuates hyperthermia-induced seizures in developing rats. Brain Dev 29, 644-648.

Garcia-Oscos, F., Salgado, H., Hall, S., Thomas, F., Farmer, G. E., Bermeo, J., Galindo, L. C., Ramirez, R. D., D'Mello, S., Rose-John, S., and Atzori, M., 2011. The Stress-Induced Cytokine Interleukin-6 Decreases the Inhibition/Excitation Ratio in the Rat Temporal Cortex via Trans-Signaling. Biol Psychiatry 71, 574-82

Haginoya, K., Noguchi, R., Zhao, Y., Munakata, M., Yokoyama, H., Tanaka, S., Hino-Fukuyo, N., Uematsu, M., Yamamoto, K., Takayanagi, M., Iinuma, K., and Tsuchiya, S., 2009. Reduced levels of interleukin-1 receptor antagonist in the cerebrospinal fluid in patients with West syndrome. Epilepsy Res 85, 314-317.

Hopkins, S. J., and Rothwell, N. J., 1995. Cytokines and the nervous system. I: Expression and recognition. Trends Neurosci 18, 83-88.

Hu, S., Sheng, W. S., Ehrlich, L. C., Peterson, P. K., and Chao, C. C., 2000. Cytokine effects on glutamate uptake by human astrocytes. Neuroimmunomodulation 7, 153-159.

Hulkkonen, J., Koskikallio, E., Rainesalo, S., Keranen, T., Hurme, M., and Peltola, J., 2004. The balance of inhibitory and excitatory cytokines is differently regulated in vivo and in vitro among therapy resistant epilepsy patients. Epilepsy Res 59, 199-205.

Ichiyama, T., Okada, K., Lipton, J. M., Matsubara, T., Hayashi, T., and Furukawa, S., 2000. Sodium valproate inhibits production of TNF-alpha and IL-6 and activation of NF-kappaB. Brain Res 857, 246-251.

Iyer, A., Zurolo, E., Spliet, W. G., van Rijen, P. C., Baayen, J. C., Gorter, J. A., and Aronica, E., 2010. Evaluation of the innate and adaptive immunity in type I and type II focal cortical dysplasias. Epilepsia 51, 1763-1773.

Jeong, E. A., Jeon, B. T., Shin, H. J., Kim, N., Lee, D. H., Kim, H. J., Kang, S. S., Cho, G. J., Choi, W. S., and Roh, G. S., 2011. Ketogenic diet-induced peroxisome proliferator-activated receptor-gamma activation decreases neuroinflammation in the mouse hippocampus after kainic acid-induced seizures. Exp Neurol 232, 195-202.

Kalueff, A. V., Lehtimaki, K. A., Ylinen, A., Honkaniemi, J., and Peltola, J., 2004. Intranasal administration of human IL-6 increases the severity of chemically induced seizures in rats. Neurosci Lett 365, 106-110.

Kamikawa, H., Hori, T., Nakane, H., Aou, S., and Tashiro, N., 1998. IL-1beta increases norepinephrine level in rat frontal cortex: involvement of prostanoids, NO, and glutamate. Am J Physiol 275, R803-810.

Kim do, Y., Davis, L. M., Sullivan, P. G., Maalouf, M., Simeone, T. A., van Brederode, J., and Rho, J. M., 2007. Ketone bodies are protective against oxidative stress in neocortical neurons. J Neurochem 101, 1316-1326.

Lehtimaki, K., Keranen, T., Huhtala, H., Hurme, M., Ollikainen, J., Honkaniemi, J., Palmio, J., and Peltola, J., 2004. Regulation of IL-6 system in cerebrospinal fluid and serum compartments by seizures: the effect of seizure type and duration. J Neuroimmunol 152, 121-125.

Lehtimaki, K., Keranen, T., Huuhka, M., Palmio, J., Hurme, M., Leinonen, E., and Peltola, J., 2008. Increase in plasma proinflammatory cytokines after electroconvulsive therapy in patients with depressive disorder. J Ect 24**,** 88-91.

Lehtimaki, K., Keranen, T., Palmio, J., Makinen, R., Hurme, M., Honkaniemi, J., and Peltola, J., 2007. Increased plasma levels of cytokines after seizures in localization-related epilepsy. Acta Neurol Scand 116**,** 226-230.

Lehtimaki, K., Keranen, T., Palmio, J., and Peltola, J., 2010. Levels of IL-1beta and IL-1ra in cerebrospinal fluid of human patients after single and prolonged seizures. Neuroimmunomodulation 17**,** 19-22.

Lehtimaki, K., Liimatainen, S., Peltola, J., and Arvio, M., 2011. The serum level of interleukin-6 in patients with intellectual disability and refractory epilepsy. Epilepsy Res 95**,** 184-187.

Levite, M., 2008. Neurotransmitters activate T-cells and elicit crucial functions via neurotransmitter receptors. Curr Opin Pharmacol 8**,** 460-471.

Liimatainen, S., Fallah, M., Kharazmi, E., Peltola, M., and Peltola, J., 2009. Interleukin-6 levels are increased in temporal lobe epilepsy but not in extra-temporal lobe epilepsy. J Neurol 256**,** 796-802.

Liu, H., Prayson, R. A., Estes, M. L., Drazba, J. A., Barnett, G. H., Bingaman, W., Liu, J., Jacobs, B. S., and Barna, B. P., 2000. In vivo expression of the interleukin 4 receptor alpha by astrocytes in epilepsy cerebral cortex. Cytokine 12**,** 1656-1661.

Liu, Z., Wang, Q. W., Wang, F. L., and Yang, L. Z., 2001. Serum cytokine levels are altered in patients with West syndrome. Brain Dev 23**,** 548-551.

Lu, Y., Xue, T., Yuan, J., Li, Y., Wu, Y., Xi, Z., Xiao, Z., Chen, Y., and Wang, X., 2009. Increased expression of TGFbeta type I receptor in brain tissues of patients with temporal lobe epilepsy. Clin Sci (Lond) 117**,** 17-22.

Majoie, H. J., Rijkers, K., Berfelo, M. W., Hulsman, J. A., Myint, A., Schwarz, M., and Vles, J. S., 2011. Vagus nerve stimulation in refractory epilepsy: effects on pro- and anti-inflammatory cytokines in peripheral blood. Neuroimmunomodulation 18**,** 52-56.

Makis, A. C., Tzoufi, M., Kateri, M. D., Bourantas, K. L., and Papadopoulou, Z. L., 2005. Valproate-induced eosinophilia in children with epilepsy: role of interleukin-5. J Child Neurol 20**,** 150-152.

Maldonado, M., Baybis, M., Newman, D., Kolson, D. L., Chen, W., McKhann, G., 2nd, Gutmann, D. H., and Crino, P. B., 2003. Expression of ICAM-1, TNF-alpha, NF kappa B, and MAP kinase in tubers of the tuberous sclerosis complex. Neurobiol Dis 14**,** 279-290.

Mathieu, O., Picot, M. C., Gelisse, P., Breton, H., Demoly, P., and Hillaire-Buys, D., 2011. Effects of carbamazepine and metabolites on IL-2, IL-5, IL-6, IL-10 and IFN-gamma secretion in epileptic patients: the influence of co-medication. Pharmacol Rep 63**,** 86-94.

Moynagh, P. N., Williams, D. C., and O'Neill, L. A., 1993. Interleukin-1 activates transcription factor NF kappa B in glial cells. Biochem J 294 (Pt 2)**,** 343-347.

Muller, M., Fontana, A., Zbinden, G., and Gahwiler, B. H., 1993. Effects of interferons and hydrogen peroxide on CA3 pyramidal cells in rat hippocampal slice cultures. Brain Res 619**,** 157-162.

Nowak, M., Bauer, S., Haag, A., Cepok, S., Todorova-Rudolph, A., Tackenberg, B., Norwood, B., Oertel, W. H., Rosenow, F., Hemmer, B., and Hamer, H. M., 2011. Interictal alterations of cytokines and leukocytes in patients with active epilepsy. Brain Behav Immun 25**,** 423-428.

Pacifici, R., Paris, L., Di Carlo, S., Bacosi, A., Pichini, S., and Zuccaro, P., 1995. Cytokine production in blood mononuclear cells from epileptic patients. Epilepsia 36**,** 384-387.

Panegyres, P. K., 1998. The effects of excitotoxicity on the expression of the amyloid precursor protein gene in the brain and its modulation by neuroprotective agents. J Neural Transm 105**,** 463-478.

Pedersen, B. K., and Hoffman-Goetz, L., 2000. Exercise and the immune system: regulation, integration, and adaptation. Physiol Rev 80**,** 1055-1081.

Peltola, J., Hurme, M., Miettinen, A., and Keranen, T., 1998. Elevated levels of interleukin-6 may occur in cerebrospinal fluid from patients with recent epileptic seizures. Epilepsy Res 31**,** 129-133.

Peltola, J., Palmio, J., Korhonen, L., Suhonen, J., Miettinen, A., Hurme, M., Lindholm, D., and Keranen, T., 2000. Interleukin-6 and interleukin-1 receptor antagonist in cerebrospinal fluid from patients with recent tonic-clonic seizures. Epilepsy Res 41**,** 205-211.

Rahimian, V., and Jyonouchi, H., An IL-1 receptor antagonist (anakinra) as a therapeutic option for treatment-resistant seizures (TRS). 2011 Annual Meeting of the American College of Allergy, Asthma and Immunology Boston, 2011, pp. A89.

Ravizza, T., Boer, K., Redeker, S., Spliet, W. G., van Rijen, P. C., Troost, D., Vezzani, A., and Aronica, E., 2006. The IL-1beta system in epilepsy-associated malformations of cortical development. Neurobiol Dis 24**,** 128-143.

Ravizza, T., Gagliardi, B., Noe, F., Boer, K., Aronica, E., and Vezzani, A., 2008. Innate and adaptive immunity during epileptogenesis and spontaneous seizures: evidence from experimental models and human temporal lobe epilepsy. Neurobiol Dis 29**,** 142-160.

Sander, J. W., 2003. The epidemiology of epilepsy revisited. Curr Opin Neurol 16**,** 165-170.

Sheng, J. G., Boop, F. A., Mrak, R. E., and Griffin, W. S., 1994. Increased neuronal beta-amyloid precursor protein expression in human temporal lobe epilepsy: association with interleukin-1 alpha immunoreactivity. J Neurochem 63**,** 1872-1879.

Shiah, I. S., Yatham, L. N., Yeh, C. B., and Ravindran, A. V., 2005. Effect of valproate on plasma levels of interleukin-6 in healthy male humans. Int Clin Psychopharmacol 20**,** 295-298.

Shiihara, T., Miyashita, M., Yoshizumi, M., Watanabe, M., Yamada, Y., and Kato, M., 2010. Peripheral lymphocyte subset and serum cytokine profiles of patients with West syndrome. Brain Dev 32**,** 695-702.

Shu, H. F., Zhang, C. Q., Yin, Q., An, N., Liu, S. Y., and Yang, H., 2010. Expression of the interleukin 6 system in cortical lesions from patients with tuberous sclerosis complex and focal cortical dysplasia type IIb. J Neuropathol Exp Neurol 69**,** 838-849.

Sinha, S., Patil, S. A., Jayalekshmy, V., and Satishchandra, P., 2008. Do cytokines have any role in epilepsy? Epilepsy Res 82**,** 171-176.

Stellwagen, D., Beattie, E. C., Seo, J. Y., and Malenka, R. C., 2005. Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-alpha. J Neurosci 25**,** 3219-3228.

Stoll, G., and Bendszus, M., 2009. Imaging of inflammation in the peripheral and central nervous system by magnetic resonance imaging. Neuroscience 158**,** 1151-1160.

Szelenyi, J., 2001. Cytokines and the central nervous system. Brain Res Bull 54**,** 329-338.

Tekgul, H., Polat, M., Tosun, A., Serdaroglu, G., Kutukculer, N., and Gokben, S., 2006. Cerebrospinal fluid interleukin-6 levels in patients with West syndrome. Brain Dev 28**,** 19-23.

Tracey, K. J., 2007. Physiology and immunology of the cholinergic antiinflammatory pathway. J Clin Invest 117**,** 289-296.

Varella, P. P., Santiago, J. F., Carrete, H., Jr., Higa, E. M., Yacubian, E. M., Centeno, R. S., Caboclo, L. O., Castro Neto, E. F., Canzian, M., Amado, D., Cavalheiro, E. A., and Naffah-Mazzacoratti Mda, G., 2011. Relationship between fluid-attenuated inversion-recovery (FLAIR) signal intensity and inflammatory mediator's levels in the hippocampus of patients with temporal lobe epilepsy and mesial temporal sclerosis. Arq Neuropsiquiatr 69**,** 91-99.

Verrotti, A., Basciani, F., Trotta, D., Greco, R., Morgese, G., and Chiarelli, F., 2001. Effect of anticonvulsant drugs on interleukins-1, -2 and -6 and monocyte chemoattractant protein-1. Clin Exp Med 1**,** 133-136.

Vezzani, A., Conti, M., De Luigi, A., Ravizza, T., Moneta, D., Marchesi, F., and De Simoni, M. G., 1999. Interleukin-1beta immunoreactivity and microglia are enhanced in the rat hippocampus by focal kainate application: functional evidence for enhancement of electrographic seizures. J Neurosci 19**,** 5054-5065.

Vezzani, A., and Granata, T., 2005. Brain inflammation in epilepsy: experimental and clinical evidence. Epilepsia 46**,** 1724-1743.

Vezzani, A., Moneta, D., Conti, M., Richichi, C., Ravizza, T., De Luigi, A., De Simoni, M. G., Sperk, G., Andell-Jonsson, S., Lundkvist, J., Iverfeldt, K., and Bartfai, T., 2000. Powerful anticonvulsant action of IL-1 receptor antagonist on intracerebral injection and astrocytic overexpression in mice. Proc Natl Acad Sci U S A 97**,** 11534-11539.

Wei, M., Li, L., Meng, R., Fan, Y., Liu, Y., Tao, L., Liu, X., and Wu, C., 2010. Suppressive effect of diazepam on IFN-gamma production by human T cells. Int Immunopharmacol 10**,** 267-271.

Wrona, D., 2006. Neural-immune interactions: an integrative view of the bidirectional relationship between the brain and immune systems. J Neuroimmunol 172**,** 38-58.

Wu, Y., Wang, X., Mo, X., Xi, Z., Xiao, F., Li, J., Zhu, X., Luan, G., Wang, Y., Li, Y., and Zhang, J., 2008. Expression of monocyte chemoattractant protein-1 in brain tissue of patients with intractable epilepsy. Clin Neuropathol 27**,** 55-63.

Wunder, A., Klohs, J., and Dirnagl, U., 2009. Non-invasive visualization of CNS inflammation with nuclear and optical imaging. Neuroscience 158**,** 1161-1173.

Yamamoto, A., Schindler, C. K., Murphy, B. M., Bellver-Estelles, C., So, N. K., Taki, W., Meller, R., Simon, R. P., and Henshall, D. C., 2006. Evidence of tumor necrosis factor receptor 1 signaling in human temporal lobe epilepsy. Exp Neurol 202**,** 410-420.

Yamanaka, G., Kawashima, H., Oana, S., Ishida, Y., Miyajima, T., Kashiwagi, Y., and Hoshika, A., 2010. Increased level of serum interleukin-1 receptor antagonist subsequent to resolution of clinical symptoms in patients with West syndrome. J Neurol Sci 298**,** 106-109.

Yang, K. D., Liou, W. Y., Lee, C. S., Chu, M. L., and Shaio, M. F., 1992. Effects of phenobarbital on leukocyte activation: membrane potential, actin polymerization, chemotaxis, respiratory burst, cytokine production, and lymphocyte proliferation. J Leukoc Biol 52**,** 151-156.

Yuhas, Y., Shulman, L., Weizman, A., Kaminsky, E., Vanichkin, A., and Ashkenazi, S., 1999. Involvement of tumor necrosis factor alpha and interleukin-1beta in enhancement of pentylenetetrazole-induced seizures caused by Shigella dysenteriae. Infect Immun 67**,** 1455-1460.

Zhou, X., Fragala, M. S., McElhaney, J. E., and Kuchel, G. A., 2010. Conceptual and methodological issues relevant to cytokine and inflammatory marker measurements in clinical research. Curr Opin Clin Nutr Metab Care 13**,** 541-547.

Chapter 3

Brain cytokine levels in patients with refractory temporal lobe epilepsy

M. Aalbers, K. Rijkers, J. Vles, J. Dings, O. Schijns, A. Kessels, G. Hoogland

Submitted in revised form

Abstract

In recent years, inflammation has been implicated in various epilepsies, including temporal lobe epilepsy (TLE). However, it is unclear to what extent pro-inflammatory changes are caused by the underlying histopathology. Therefore we aimed to study the expression of a broad array of cytokines in hippocampi from TLE patients with hippocampal sclerosis (HS).

Sclerotic hippocampi from 19 TLE patients were compared with histologically normal cortex (available in 6 out of 19 patients) and with histologically normal hippocampi from non-HS TLE patients without HS (n=4). The following cytokines and receptors were simultaneously assessed by a multiplex immuno-assay: IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-1ra, MCP-1, MIP-1α, MIP-1β, TNF-α, IL-1RI, and TNF-RII. Furthermore, we evaluated whether cytokine levels were related to the extent of HS, age of onset, epilepsy duration, history of febrile convulsions, family history of epilepsy, or side of seizure onset.

Only IL-1α and IL-1ra were significantly lower in HS than in histologically normal cortex. There was no relation between cytokine levels and any of the above-mentioned clinical parameters.

These results suggest that HS does not differentially determine cytokine expression.

Introduction

In recent years, inflammatory processes have been implicated in the pathophysiology of many neurological disorders, including epilepsy. Inflammation clearly plays a role in epilepsies such as autoimmune epilepsy and Rasmussen encephalitis. Yet, temporal lobe epilepsy (TLE) has recently also been associated with neuroinflammation (Vezzani, et al., 2011). Increased levels of interleukin (IL)-1, IL-6, and monocyte chemotactic protein-1 (MCP-1) have been shown in resected brain tissue of patients with refractory TLE (Choi, et al., 2009, Ravizza, et al., 2008, Sheng, et al., 1994, Varella, et al., 2011, Wu, et al., 2008). So far, most of these studies limited their analyses to just a few cytokines. However, the physiological outcome of an inflammatory process results from the interaction between many different cytokines. Hence, analysis of several cytokines would be more informative.

Furthermore, it is unclear whether the increased levels of IL-1, IL-6, and MCP-1 are intrinsic to epilepsy or whether they result from the underlying pathology. The most common pathology associated with TLE is hippocampal sclerosis (HS) that is characterized by neuronal cell loss and gliosis. Some studies suggest that the increased cytokine expression is associated with this specific neuropathology. For instance, hippocampal expression of nuclear factor kappa B, a transcription factor involved in inflammatory processes, was found to be increased in patients with HS compared to that in non-HS patients (Crespel, et al., 2002). Similarly, hippocampal levels of the pro-inflammatory cytokine IL-1β were increased in patients with HS, but not in patients with TLE without HS (Ravizza, et al., 2008).

Therefore, we aimed to establish whether HS influences cytokine expression by comparing a broad array of cytokines in TLE patients with and without HS.

Methods

Patients

We examined nineteen sclerotic hippocampi that were intraoperatively obtained at Maastricht University Medical Centre between January 2010 and February 2012 (mean age ± SEM 44.6±3.54 years; 9 males). All patients suffered from medically refractory TLE (Kwan, et al., 2010). Extensive presurgical evaluation included video-EEG monitoring, neuropsychological testing, MRI, and FDG-PET imaging as needed.

Tissue collection

Hippocampi were resected *en bloc* and divided in two parts. One part was fixed in paraformaldehyde and used for routine histopathological evaluation. The other part was immediately frozen on dry ice and stored at -80°C until further analysis. Sclerosis

was graded according to Wyler et al., ranging from I (mild HS) to IV (severe HS) (Wyler, et al., 1992).
Two types of control tissue were collected. The first type consisted of non-sclerotic hippocampus, which was obtained from TLE patients undergoing an amygdala-hippocampectomy for focal refractory epilepsy (mean age ± SEM 43.4±10.1 years; 3 males, 1 female). Second, in six out of the nineteen patients with HS, histologically normal neocortex was obtained (mean age ± SEM age 42.2±7.3 years; 3 males, 3 females). This group served as an internal control as this tissue was exposed to similar seizure activity in the absence of histological changes. Informed consent was obtained for the use of brain tissue and access to medical records for research purposes. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki.

Tissue processing and cytokine analysis

Biopsies were homogenized in Greenberger Lysis Buffer (100 µg: 1 ml) containing 150 mM NaCl, 15 mM Tris, 1 mM $MgCl(H2O)_6$, 1 mM $CaCl_2(H_2O)_2$, 1% Triton, and a cocktail of protease inhibitors (Roche Diagnostics Nederland B.V., Almere, the Netherlands). Concentrations of IL-1α, IL-1β, soluble IL-1-receptor type I (IL-1RI), IL-6, IL-8, IL-10, IL-1 receptor antagonist (IL-1ra), MCP-1, macrophage inflammatory protein-1alpha (MIP-1α), macrophage inflammatory protein-1beta (MIP-1β), tumor necrosis factor-alpha (TNF-α), and of soluble TNF-α -receptor type II (TNF-RII) were simultaneously measured by a multiplex immunoassay according to the manufacturers instructions (Millipore, Massachusetts, USA). Fluorescent signal was measured using the Luminex LX200 platform (Luminex corporation, Austin, USA). Concentrations were estimated based on calibration curves that were obtained from the respective recombinant proteins diluted in lysis buffer with help of Milliplex Analyst software using a five-parameter logistic curve-fitting method. Cytokine concentrations were normalized to total protein concentrations. The lower limit of detection was 3.2 pg/ml for all cytokines and chemokines, 24.4 pg/ml for IL-1RI, and 12.2 pg/ml for TNF-RII. For statistical analysis, concentrations below the detection limit were converted to a value of 0.5 times the lowest value of the calibration curve (Majoie, et al., 2011, Ricker, et al., 2011).

Statistical analysis

Hippocampal cytokine levels were compared between patients with and without HS by Mann-Whitney U test. In patients from whom neocortex was available, cytokine levels were compared between sclerotic hippocampus and histopathologically normal neocortex by Wilcoxon signed rank test.
To assess whether the extent of HS was related to cytokine concentration, we evaluated whether the Wyler grade correlated with cytokine levels (Kendall's tau). We performed a similar analysis for age at onset and epilepsy duration. Finally, we

compared cytokine levels between patients with left and right-sided seizure focus, positive and negative family history, and with and without a history of febrile seizures within the nineteen patients with HS by Mann-Whitney U test. A p-value <0.05 was considered statistically significant.

Results

Clinical data

Clinical characteristics of all patients are presented in Table 3.1. Thirteen patients (57%) had experienced their first seizures during childhood or adolescence. All but two patients were on polytherapy. The most frequent administered antiepileptic drugs included clobazepam (n=12), lamotrigine (n=11), levetiracetam (n=8), oxcarbazepine (n=7), and carbamazepine (n=7).

Table 3.1 Clinical characteristics of patients.

	HS		Non-HS
	Hippocampus	Cortex	Hippocampus
Male / female	9 / 10	3 / 3	3 / 1
Mean age at seizure onset (SEM), years	19.7 (± 3.9)	16.8(± 7.4)	22.5 (± 10.6)
Mean epilepsy duration (SEM), years	25.0 (± 4.6)	25.5 (± 10.1)	20.9 (± 9.8)
Seizure onset (left / right)	8 / 11	2 / 4	0 / 4
Positive family history	3	2	0
History of febrile convulsions	6	2	0
Mean overall number of AEDs used (SEM), years	6 (± 1)	5(± 1)	8 (± 2)

AED= antiepileptic drug.

Cytokine (receptor) levels

Hippocampi from HS cases expressed similar cytokine and receptor levels as hippocampi from non-HS cases.

Within the HS-patient group hippocampal expression of IL-1α and IL-1ra was significantly lower than expression in histologically normal neocortex ($z=-1.992$, $p<0.05$ and $z=-2.201$, $p<0.05$, respectively; Figure 3.1).

Finally, the extent of the HS did not influence cytokine or receptor concentrations, as the levels did not significantly differ between the different Wyler grades. No differences or correlations were observed between cytokine and receptor levels and age at onset, epilepsy duration, left or right-sided seizure focus, family history of epilepsy, or history of febrile seizures.

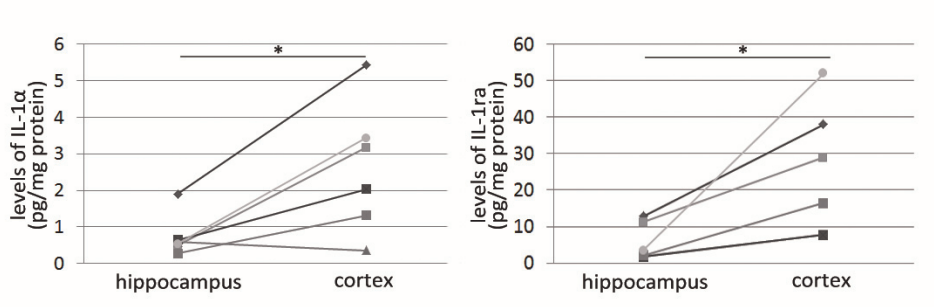


Figure 3.1 Individually matched hippocampal and cortical levels in temporal lobe epilepsy patients with hippocampal sclerosis. *p<0.05.

Discussion

This study demonstrates that sclerotic hippocampi from TLE patients express lower levels of IL-1α and IL-1ra than histologically normal neocortex. Both IL-1α and IL-1ra belong to a subgroup of interleukins called the IL-1 family. IL-1α induces inflammation by binding to the IL-1RI and this action is antagonised by IL-1ra (Dinarello, 1996). The relatively lower levels of hippocampal IL-1α might actually represent higher cortical levels, because the only previous study that evaluated IL-1α in TLE-patients demonstrated an increase of IL-1α in layer V of the temporal lobe of TLE-patients compared to autopsy material (Sheng, et al., 1994). Moreover, IL-1α levels did not differ between sclerotic and non-sclerotic hippocampi. Levels of IL-1ra were lower in sclerotic hippocampi than in histologically normal neocortex. These lower levels suggest a decreased inhibition of IL-1 signalling. Although no studies on IL-1ra in brain tissue of TLE-patients have been performed, a relatively low expression of IL-1ra was also observed in patients with malformation of cortical development, another frequent cause of refractory epilepsy (Ravizza, et al., 2006).

Remarkably, we did not find any increase in pro-inflammatory cytokines in HS. Apparently in our study HS is not associated with a pro-inflammatory status. This contrasts with the findings of Ravizza et al and Crespel et al (Crespel, et al., 2002, Ravizza, et al., 2008). Possibly this discrepancy results from the different techniques that were used: whereas the other studies obtained qualitative data from immunohistochemistry, we applied a multiplex assay, which may also use different antibodies. Furthermore, unlike the other studies we also included patients with mild to moderate forms of sclerosis. However, this probably does not influence the results, because Wyler grades did not correlate with cytokine levels.

Our results indicate that the lower levels of IL-1α and IL-1ra are a specific characteristic of the seizure focus and not merely result from seizure activity. After all, neocortical control tissue has been exposed to seizure activity as well. In line with our results, previous studies have shown increased cytokine levels in epileptogenic lesions but not

in the cortex surrounding these lesions (Boer, et al., 2008, Maldonado, et al., 2003, Ravizza, et al., 2006), suggesting that seizure activity in itself is not necessarily associated with cytokine changes.

Some limitations of the present study should be mentioned. The relatively small number of non-sclerotic hippocampi available might have limited the possibility to detect a significant difference. Furthermore, ideally freshly collected cortical samples from non-neurological control subjects would have been included as a third control group to compare cytokine levels in HS to basal cytokine levels. As these samples are clearly not at hand, other studies have used autopsy brain material as an alternative control. Since previous papers found similar cytokine levels in brain tissue of healthy controls and histologically normal brain tissue of epilepsy patients (Ravizza, et al., 2006, Ravizza, et al., 2008), we believe that inclusion of this extra group would not alter our conclusion. In other words, hippocampal cytokine levels might not be upregulated at all in our patient group. Future studies should therefore evaluate whether inflammation is a necessary characteristic of TLE.

Conclusion

IL-1alpha and IL-1ra expression are significantly lower in sclerotic hippocampus than in normal cortex, while levels of the ten other cytokine (receptors) that we analysed, remained unaltered. These findings indicate that HS is not associated with a pro-inflammatory state.

References

Boer, K., Jansen, F., Nellist, M., Redeker, S., van den Ouweland, A. M., Spliet, W. G., van Nieuwenhuizen, O., Troost, D., Crino, P. B., and Aronica, E., 2008. Inflammatory processes in cortical tubers and subependymal giant cell tumors of tuberous sclerosis complex. Epilepsy Res 78**,** 7-21.

Choi, J., Nordli, D. R., Jr., Alden, T. D., DiPatri, A., Jr., Laux, L., Kelley, K., Rosenow, J., Schuele, S. U., Rajaram, V., and Koh, S., 2009. Cellular injury and neuroinflammation in children with chronic intractable epilepsy. J Neuroinflammation 6**,** 38.

Crespel, A., Coubes, P., Rousset, M. C., Brana, C., Rougier, A., Rondouin, G., Bockaert, J., Baldy-Moulinier, M., and Lerner-Natoli, M., 2002. Inflammatory reactions in human medial temporal lobe epilepsy with hippocampal sclerosis. Brain Res 952**,** 159-169.

Dinarello, C. A., 1996. Biologic basis for interleukin-1 in disease. Blood 87**,** 2095-2147.

Kwan, P., Arzimanoglou, A., Berg, A. T., Brodie, M. J., Allen Hauser, W., Mathern, G., Moshe, S. L., Perucca, E., Wiebe, S., and French, J., 2010. Definition of drug resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAE Commission on Therapeutic Strategies. Epilepsia 51**,** 1069-1077.

Majoie, H. J., Rijkers, K., Berfelo, M. W., Hulsman, J. A., Myint, A., Schwarz, M., and Vles, J. S., 2011. Vagus nerve stimulation in refractory epilepsy: effects on pro- and anti-inflammatory cytokines in peripheral blood. Neuroimmunomodulation 18**,** 52-56.

Maldonado, M., Baybis, M., Newman, D., Kolson, D. L., Chen, W., McKhann, G., 2nd, Gutmann, D. H., and Crino, P. B., 2003. Expression of ICAM-1, TNF-alpha, NF kappa B, and MAP kinase in tubers of the tuberous sclerosis complex. Neurobiol Dis 14**,** 279-290.

Ravizza, T., Boer, K., Redeker, S., Spliet, W. G., van Rijen, P. C., Troost, D., Vezzani, A., and Aronica, E., 2006. The IL-1beta system in epilepsy-associated malformations of cortical development. Neurobiol Dis 24**,** 128-143.

Ravizza, T., Gagliardi, B., Noe, F., Boer, K., Aronica, E., and Vezzani, A., 2008. Innate and adaptive immunity during epileptogenesis and spontaneous seizures: evidence from experimental models and human temporal lobe epilepsy. Neurobiol Dis 29**,** 142-160.

Ricker, L. J., Kijlstra, A., Kessels, A. G., de Jager, W., Liem, A. T., Hendrikse, F., and La Heij, E. C., 2011. Interleukin and growth factor levels in subretinal fluid in rhegmatogenous retinal detachment: a case-control study. PLoS One 6**,** e19141.

Sheng, J. G., Boop, F. A., Mrak, R. E., and Griffin, W. S., 1994. Increased neuronal beta-amyloid precursor protein expression in human temporal lobe epilepsy: association with interleukin-1 alpha immunoreactivity. J Neurochem 63**,** 1872-1879.

Varella, P. P., Santiago, J. F., Carrete, H., Jr., Higa, E. M., Yacubian, E. M., Centeno, R. S., Caboclo, L. O., Castro Neto, E. F., Canzian, M., Amado, D., Cavalheiro, E. A., and Naffah-Mazzacoratti Mda, G., 2011. Relationship between fluid-attenuated inversion-recovery (FLAIR) signal intensity and inflammatory mediator's levels in the hippocampus of patients with temporal lobe epilepsy and mesial temporal sclerosis. Arq Neuropsiquiatr 69**,** 91-99.

Vezzani, A., French, J., Bartfai, T., and Baram, T. Z., 2011. The role of inflammation in epilepsy. Nat Rev Neurol 7**,** 31-40.

Wu, Y., Wang, X., Mo, X., Xi, Z., Xiao, F., Li, J., Zhu, X., Luan, G., Wang, Y., Li, Y., and Zhang, J., 2008. Expression of monocyte chemoattractant protein-1 in brain tissue of patients with intractable epilepsy. Clin Neuropathol 27**,** 55-63.

Wyler, A. R., Dohan, F. C., Schweitzer, J. B., and Berry III, A. D., 1992. A Grading System for Mesial Temporal Pathology (Hippocampal Sclerosis) from Anterior Temporal Lobectomy. Journal of Epilepsy 5, 220-225.

Chapter 4

Experimental epilepsy without neuroinflammation

M. Aalbers, K. Rijkers, M. Majoie, M. de Baets, A. Kessels, J. Vles, G. Hoogland

Submitted in revised form

Abstract

Recent evidence of neuroinflammation in human temporal lobe epilepsy (TLE) suggests that inflammatory mediators play a role in epilepsy. However, it is still unclear to what extent inflammation in TLE is caused by the underlying neuropathological changes. Therefore, we studied inflammatory processes in an animal model for TLE without overt neuronal cell loss.

Amygdala kindled rats were compared to sham rats that were implanted with an electrode, but not stimulated. Hippocampi were analyzed for I) activated astrocytes (GFAP), microglia (OX-42) and neuronal cell loss (NeuN) by immunohistochemistry (n=5 per group); II) cytokine levels (IL-1α, IL-1β, IL-6, IL-10, IL-18, MIP-1α, and TNF-α) by multiplex assay (n=8 per group); and III) phosphorylated NMDA-receptors by Western blot (n=10-11 per group).

Kindling was associated with increased GFAP expression. We did not observe overt microglia activation or neuronal cell loss after amygdala kindling. Cytokine levels and phosphorylation of NMDA-receptor were unaltered as well.

These results suggest that inflammation, and in particular upregulation of pro-inflammatory brain cytokines, is not required for the development of amygdala kindling.

Introduction

Epilepsy is one of the most common neurological disorders, affecting up to 1% of the world's population (Sander, 2003). Despite this high incidence, its pathophysiology has only been partly elucidated. Recent clinical an preclinical evidence suggests that brain inflammation plays a key role in epilepsy (Vezzani, et al., 2011).

Inflammatory mediators such as cytokines are known to influence neuronal excitability, thereby contributing to seizure generation. For instance, interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α) can increase extracellular glutamate concentrations by increasing glutamate release and inhibiting astrocytic glutamate uptake (Bezzi, et al., 2001, Hu, et al., 2000, Kamikawa, et al., 1998). Moreover, cytokines and other inflammatory mediators can initiate several cascades that may contribute to seizure generation. An example of such a downstream effect is phosphorylation of the NR2B subunit of the N-methyl-D-aspartate receptor (NMDAR). This posttranslational modification results in enhanced functioning of this receptor and thereby in increased neuronal excitability (Maroso, et al., 2011). Receptor activation by cytokines also induces slower changes by affecting transcriptional pathways that result in structural and functional changes of glial and neuronal networks (Moynagh, et al., 1993). Finally chemokines, a family of chemotactic cytokines, can contribute to epileptic seizures by inducing blood brain barrier (BBB) leakage and by directly altering neuronal activity (Fabene, et al., 2010).

Most studies that imply cytokines in epilepsy have been performed in animal models that are characterized by extensive neuronal injury such as the status epilepticus (SE) model. However, most types of epilepsy in humans, including the most common type, temporal lobe epilepsy (TLE), are not associated with such extensive neuronal injury. Therefore, the question rises whether neuroinflammation is also present in the absence of ongoing neuronal injury. Models that lack extensive neuronal injury may therefore provide additional insights into the occurrence of inflammation in epilepsy. An example of such a model is the amygdala kindling (AK) model. Neuroinflammation following amygdala kindled seizures has previously been demonstrated at the level of gene transcription (Plata-Salaman, et al., 2000), yet data on protein levels are scarce (Shandra, et al., 2002, Wood, et al., 2011). These studies mostly analyzed only one or a few cytokines, whereas analysis of a broad array of cytokines better reflects the complex inflammatory response. The purpose of this study was therefore to evaluate the neuroinflammatory response following AK. In addition to the analysis of a cytokine panel, we assessed a downstream effect of cytokine-induced receptor activation, i.e., phosphorylation of the NMDAR.

Methods

Animals

Male 10 weeks old Sprague-Dawley rats, purchased from Harlan (Horst, The Netherlands) were kept under controlled standard conditions (21±2°C ambient temperature, a 12 hour light/dark cycle, background noise provided by radio, and food and water available *ad libitum*). The animals adjusted to their housing conditions for one week before surgery. All experimental procedures were approved by the animal ethics committee of Maastricht University and were in accordance with international standards as defined by the European Communities Council Directive of November 24th 1986.

Surgery

Implantation was performed as described previously (Rijkers, et al., 2010). Briefly, rats were operated under general isoflurane anaesthesia (5% for induction and 2.5% for maintenance). Additionally, rats received 0.1 ml buprenorfine hydrochloride (Temgesic®, Schering-Plough Inc., Amstelveen, The Netherlands) thirty minutes before the surgery to reduce peroperative pain. Fifty-two rats were implanted with a custom made electrode that consisted of a bipolar platinum/iridium needle with a 200 μm diameter tip (Department of Instrument Development, Engineering & Evaluation of Maastricht University). This stimulating/recording electrode was implanted in the left basolateral amygdala (BLA, coordinates relative to bregma: -2.5 mm posteriorly, 4.8 mm laterally, and 9.6 mm ventrally) using a standard rat stereotact (Dual Manipulator Lab Standard Stereotact, Stoelting Inc., Wood Dale, Ill, USA). In addition, a stainless steel screw was implanted over the nasal sinus that served as ground and reference.

Amygdala kindling

Amygdala stimulation started 10 days after the surgery. Initially stimulation was performed twice daily (first stimulus between 8 and 10 am, second stimulus between 2 and 4 pm; interstimulus interval at least 6 hours) with the following stimulation parameters: 2s, 400 μA, 50 Hz, 0.2 ms block pulses. A stimulus intensity of 400 μA was chosen to assure that the intensity was above the after discharge threshold for all rats. Stimuli were delivered through a WPI Accupulser A310 connected to a WPI Stimulus Isolation Unit A360 (World Precision Instruments, Sarasota, FL, USA).
All rats were videotaped (Olympus FE-330) during delivery of the kindling stimulus and for as long as the behavioural seizure lasted. Seizure severity was evaluated offline from video-recordings by 2 blinded observers and classified according to the Racine scale (Racine, 1972).

After reaching the fully kindled state, defined as five consecutive stage five seizures, rats received one AK stimulation per day for two more weeks. Sham rats received an amygdalar electrode that was not stimulated.

(Immuno) Histochemistry

Two hours and 24 hours after the last seizure, rats (n=5 per group) received an overdose of pentobarbital (Nembutal, 0.1 mg/ kg body weight) and were then trans-cardially perfused with 50 mM ice cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 50 mM PBS. These time points were chosen in order to discriminate acute and chronic seizure effects. Brains were isolated and post-fixed in the same fixative for 90 min and then transferred to 20% sucrose in PBS for 24 h. Subsequently, the brains were immersed in -40°C isopentane for 3 min and stored at -80°C until immunohistochemistry was performed. Horizontal 40 µm thick sections were serially cut throughout the hippocampus using a cryostat. Standard haematoxylin-eosin (Merck, Germany) staining was used to verify the location of the electrode tip.
Immunohistochemistry was carried out as described previously (Ravizza, et al., 2008). Briefly, free-floating sections were successively incubated at 4°C for 30 min in PBS supplemented with 0.4% Triton X-100 and for 15 min in PBS supplemented with 3% fetal bovine serum (FBS) and 0.1% Triton X-100. Then sections were incubated overnight at 4°C in 3% FBS in 0.1% Triton X-100 in PBS and one of the following primary antibodies: mouse anti-glial fibrillary acidic protein as a selective marker for astrocytes (GFAP, diluted 1:2500, Chemicon Int. Inc., Temecula, USA), mouse anti-CD11b as a marker for microglia-like cells (complement receptor type 3, OX-42, diluted 1:100, Serotec Ltd, Oxford, UK), or mouse anti-neuronal specific nuclear protein as a selective neuronal marker (NeuN, diluted 1:1000, Chemicon, USA). Immunoreactivity was tested by the avidin–biotin–peroxidase technique (Vectastain ABC kit, Vector, Burlingame, USA) using 3,3'-diaminobenzidine (DAB; Sigma, Munich, Germany) as chromogen. To avoid interstaining bias, sections from shams and both experimental groups were processed together.

Image analysis

Four sections per animal throughout the right ventral hippocampus were analyzed for each marker; the regions of interest were CA1, CA3, and DG. Images were captured at 20x magnification using an Olympus AX-70 microscope connected to a digital camera (F-view; Olympus, Tokyo, Japan).
For glial markers, areas of immunoreactivity were highlighted at a constant threshold using NIH ImageJ software (http://rsb.info.nih.gov/ij/). Subsequently, the percentage area above the threshold was measured and the average of the four sections for each animal was calculated (Choi, et al., 2009).

Two independent observers estimated neuronal cell loss by counting the amount of NeuN immunoreactive pyramidal cells and dentate granule cells. The number of cells was divided by the area surface to obtain a cell density. For each rat, cell density values from the two observers were averaged (Frasca, et al., 2011). Cell densities in experimental groups were expressed as percentage of the average cell density in sham animals.

Fresh frozen tissue

Rats were decapitated 2 or 24 h after the last seizure. Both hippocampi were removed, immediately frozen on dry ice and stored at -80°C. The left hippocampus was used for multiplex analysis of cytokine concentrations, the right hippocampus for Western blot analysis of the phosphorylated NMDAR.

Multiplex analysis

Hippocampi (n=8 per group) were homogenized in 1 ml Greenberger Lysis Buffer containing 150mM NaCl, 15mM Tris, 1mM $MgCl(H2O)_6$, 1mM $CaCl_2(H_2O)_2$, 1% Triton X-100, and a cocktail of protease inhibitors (Roche Diagnostics Nederland B.V., Almere, Netherlands). Concentrations of interleukin-1alpha (IL-1α), IL-1β, IL-6, IL-10, IL-18, macrophage inflammatory protein-1alpha (MIP-1α), and TNF-α were measured simultaneously according to the manufacturers instructions (Millipore, Massachusetts, USA). Concentrations were estimated using a calibration curve obtained from the respective recombinant proteins diluted in lysis buffer with help of Milliplex analyst software with a five-parameter logistic curve-fitting method. Concentrations were normalized to the total protein concentration of each sample. The lower limit of detection was 4.9 pg/ml.

Western blot

Hippocampi (n=10-11 per group) were homogenized in 1 mL buffer containing 0.32 M sucrose, 1 mM Hepes, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 0.1 mM PMSF, and a cocktail of phosphatase and protease inhibitors (Sigma-Aldrich, Zwijndrecht, The Netherlands). The protein concentration of the samples was assessed via the Bradford protein assay (Bio-Rad Protein Assay, Bio-Rad Labs, Munich, Germany). To ensure that possible differences in phosphorylation of the NR2B subunit had not been masked in total homogenates, Western blot analysis was also performed in a postsynaptic density (PSD)-enriched fraction, which was obtained using a subcellular fractionation procedure (Gardoni, et al., 2009).
Total proteins (40 and 35 µg per lane for homogenate and PSD-enriched fraction, respectively) were separated using 8% SDS-PAGE. Each sample was run in duplicate. Proteins were then transferred to a nitrocullulose membrane (Bio-Rad, CA, USA) by electroblotting (150 min, 230 mA). Subsequently membranes were blocked with 10% milk. For immunoblotting, membranes were incubated at 4°C for 24 h with rabbit anti-

phospho Tyr^{1472} NR2B (1:1000; Affinity Bioreagent Golden, CO, USA). To measure total levels of NR2B, the same membranes were stripped and incubated 24h with mouse anti-NR2B (1:1000, Santa Cruz, CA, USA). Immunoreactivity was visualized with enhanced chemiluminescence (ECL; Amersham, U.K.) Densitometric analysis of immunoblots was done to quantify changes in protein levels by Quantity One software (Bio-Rad Laboratories) using film exposures with maximal signals below the photographic saturation point. Optical density values in each sample were normalized using the corresponding amount of β-actin (Merck Millipore, Massachusetts, USA).

Statistical analysis

Statistical analysis was performed using SPSSv19 for MacOSX. In order to evaluate the effects of kindling on neuroinflammatory markers, we compared the expression of glial markers, neuronal density, cytokine concentrations, and NR2B and phosphorylated NR2B levels between sham and kindled animals by Mann Whitney U test. In order to discriminate between acute and chronic seizure effects, we also compared these inflammatory markers between rats that were sacrificed 2 and 24 hours after a seizure by Mann Whitney U test.

Results

In total, 52 rats were implanted. Five rats did not complete the experiment because of anaesthesia-related death (n=1) and loss of kindling/EEG electrode (n=4). Evaluation of the electrode location in haematoxylin-eosin stained sections showed that the electrode tip was on target in all rats used for immunohistochemistry.

Glial cell activation and no neuronal cell loss

To determine whether amygdala kindled seizures resulted in glial activation, we assessed the hippocampal distribution of the astrocyte marker GFAP (Figure 4.1A) and the microglial marker OX-42 (Figure 4.1B). GFAP expression was increased in the CA1 and DG subfields of kindled rats compared to shams ($U=3.0$, $p<0.01$; $U=1.0$, $p<0.01$ respectively; Figure 4.1D). There were no significant differences in OX-42 immunoreactivity (Figure 4.1E) or neuronal cell density (Figure 4.1F), neither between kindled and sham rats, nor between animals sacrificed at 2 hour and 24 hours.

Cytokine concentrations are not altered following kindling-induced seizures

To evaluate whether kindling-induced seizures resulted in acute or chronic changes in pro- or anti-inflammatory cytokine levels, we measured cytokine and chemokine levels in hippocampal brain homogenates 2 and 24 h following the last seizure (Table 4.1).

None of the assessed markers (i.e., IL-1α, IL-1β, IL-6, IL-10, IL-18, MIP-1α, and TNF-α) were differently expressed after amygdala kindling. Similarly levels were not significantly different between the 2 h and 24 h experimental groups.

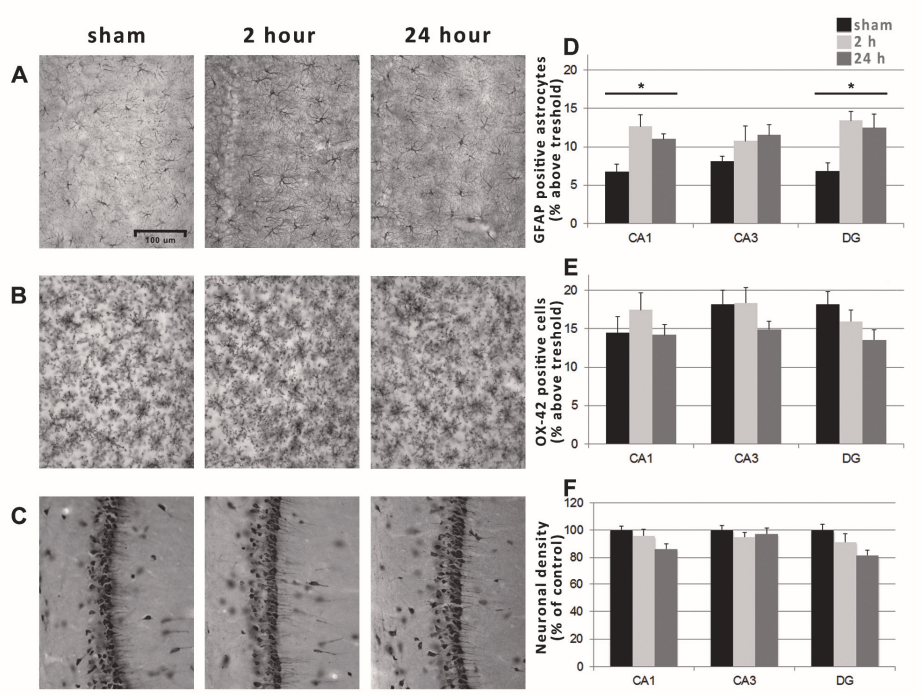


Figure 4.1 Astrocytes, microglia-like cells, and neurons in hippocampus after amygdala kindled seizures. Representative photomicrographs showing changes in GFAP-positive astrocytes (panel A), OX-42 immunoreactive microglia-like cells (panel B), and neurons (panel C) in CA1 pyramidal layer (selected as a representative hippocampal area) at various time points after amygdala kindled seizures. Note the increase of GFAP immuoreactivity in the CA1 and DG subfield of the hippocampus after amygdala kindled seizures (panel D). No significant differences in microglia-like cells or neuronal numbers were observed between sham and amygdala kindled rats (panel E and F). * $p<0.01$.

Kindling-induced seizures do not change NR2B phosphorylation

To determine if kindling-induced seizures activated cytokine-mediated pathways, we evaluated the degree of phosphorylated NR2B by Western blotting. In both fractions, i.e. total hippocampal homogenate and PSD-enriched fraction, we found no difference in the amounts of phosphorylated and total NR2B (Figure 4.2) between sham and kindled animals. Phosphorylated and total NR2B were not significantly different between rats sacrificed 2h and 24 hours after a seizure.

Table 4.1 Cytokine levels in hippocampal homogenates.

Cytokine	sham	2h	24h	p^1	p^2
IL-1α	6.4	13.2	8.5	0.08	0.28
IL-1β	29.7	29.4	22.8	0.70	0.96
IL-6	21.1	21.7	18.2	0.83	0.57
IL-10	135.3	155.4	136.4	0.74	0.88
IL-18	108.7	122.4	122.5	0.70	0.80
MIP-1α	0.4	0.7	0.5	0.08	0.20
TNF-α	3.9	4.9	4.2	0.65	0.80

Values are median concentrations in pg/mg; p^1 comparison of sham and kindled animals by Mann-Whitney test; p^2 comparison of animals sacrificed 2 and 24 hours after the last seizures by Mann-Whitney test.

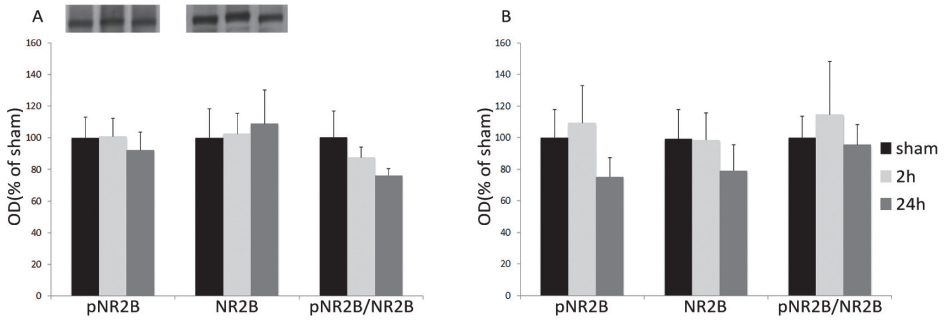


Figure 4.2 Western blot analysis of Tyr1472 phosphorylated NR2B (P-NR2B), total NR2B, and the ratio between pNR2B and NR2B. Representative bands of each protein are depicted in the upper part of panel A. Bargrams show mean±SEM of the optical density (O.D.) values of relevant bands divided by the corresponding β-actin (internal standard) in homogenate (panel A) and PSD-enriched fraction (panel B). Data are expressed as percentage of control values (sham rats).

Discussion

This study aimed to evaluate the inflammatory response following seizures that are not associated with gross neuronal cell loss, in order to determine the influence of neuronal injury on neuroinflammation. Here we present three lines of evidence that show that kindling-induced seizures are not associated with a pro-inflammatory response. Firstly, hippocampal OX-42 immunoreactivity was unaltered after amygdala kindled seizures. Second, hippocampal cytokine levels were unaltered in kindled rats. Third, phosphorylation of NR2B, a downstream effect of cytokines, was not changed after amygdala kindling. Previous studies that evaluated inflammation after perforant path and rapid kindling also found no sign of reactive microglia (Khurgel, et al., 1995,

Tooyama, et al., 2002), further supporting the idea that a neuroinflammatory response is absent in this model. Results on cytokine levels after kindling are more conflicting. Two hours after the last amygdala kindled seizure, hippocampal mRNA levels of IL-1β and TNF-α mRNA were increased in one study (Plata-Salaman, et al., 2000), but unaltered in a second study (Yi, et al., 2004). In line with our results, Wood et al. did not find any change in hippocampal protein levels of IL-1β, IL-6, IL-10, and TNF-α after rapid kindled seizures (Wood, et al., 2011). Yet, another study demonstrated increased protein levels of TNF-α after amygdala kindling (Shandra, et al., 2002). Technical issues might explain the difference in expression of TNF-α. Shandra et al determined total brain levels by ELISA, while we determined hippocampal levels by multiplex assay, which also may imply the use of different antibodies.

The absence of a pro-inflammatory response might be explained by several reasons. First of all, seizure frequency in the current design may have been too low to induce any transcriptional changes. However, a previous study that used the SE model, demonstrated upregulation of IL-1β during the chronic phase of the SE model, in which animals experience one seizure a day as well (Ravizza, et al., 2008). Furthermore, in human epilepsy, a seizure frequency of one generalized seizure per day is considered high. Therefore, it seems less likely that the seizure frequency in our design is responsible for the lack of cytokine changes.

Second, the lack of extensive neuronal cell loss, as described by others and demonstrated here by a NeuN-staining, might explain the absence of a pro-inflammatory response (Morimoto, et al., 2004, Tuunanen and Pitkanen, 2000). Indeed, upregulation of IL-1β, IL-1ra, IL-6, IL-18, and TNF-α is present in the SE model that is associated with widespread brain damage and prominent cell loss after the acute phase (Jarvela, et al., 2011, Lee, et al., 2011, Pernot, et al., 2011, Ravizza, et al., 2008, Ravizza, et al., 2005, Ryu, et al., 2010, Vezzani, et al., 2002). The importance of neuronal cell loss is further supported by the fact that after SE IL-1β was mainly expressed in regions where neurodegeneration was most pronounced (Voutsinos-Porche, et al., 2004). Furthermore, in patients with TLE, IL-1β was only increased in sclerotic hippocampal specimens, i.e. in specimens with neuronal cell loss, but not in histologically normal hippocampi (Ravizza, et al., 2008). On the other hand, even in the SE model inflammation is not always present during the chronic phase, when animals experience spontaneous seizures: some studies show an upregulation of cytokines (Bovolenta, et al., 2010, Ravizza, et al., 2008, Ryu, et al., 2010, Vezzani, et al., 2002), while others found that cytokine levels were unchanged (De Simoni, et al., 2000, Lehtimaki, et al., 2003, Pernot, et al., 2011, Rosell, et al., 2003, Vezzani, et al., 2002). These studies and our results therefore suggest that the chronic epileptic state is not necessarily associated with a pro-inflammatory response.

Even though we did not observe any signs of inflammation, GFAP immunoreactivity was significantly increased in subareas of the hippocampus. This increase likely reflects astrogliosis, which is observed both in patients with temporal lobe epilepsy and in other kindling studies (Khurgel and Ivy, 1996, Khurgel, et al., 1995, Nishio, et al., 2000).

The fact that GFAP upregulation takes place in the absence of neuronal cell loss, and in the absence of enhanced cytokine expression, suggests that activation of astrocytes is not dependent on neuronal cell loss but occurs in response to hyperactivity of neurons.

A limitation of this study is that we only sacrificed animals 2 and 24 h after a seizure. We specifically chose these time points in order to evaluate both ictal and interictal changes. We hypothesized that a post-ictal interval of 2 h would be sufficient to demonstrate a possible change in cytokine levels, because previous studies demonstrated an upregulation of IL-1β, IL-6, and TNF-α already 2-3 h after kainate or bicuculline induced seizures (Lehtimaki, et al., 2003, Vezzani, et al., 2000, Vezzani, et al., 2002). However, we cannot exclude that cytokine levels were altered at a different time point. We did not evaluate whether cytokine levels change during the kindling process and whether inflammation contributes to kindling acquisition. A previous study demonstrated that low doses of IL1-β slowed down the kindling rate (Sayyah, et al., 2005). On the other hand, blockade of IL-1β reduced epileptogenesis in other models of TLE (Maroso, et al., 2011, Ravizza, et al., 2008, Vezzani, et al., 2002). As no such studies have been performed in amygdala kindling, it would be interesting to evaluate whether blockade of cytokines influences kindling acquisition as well.

Conclusion

Inflammatory markers are not upregulated after generalized convulsive kindling-induced seizures. This suggests that inflammation is not a prerequisite for the epilepsy-prone state in this particular epilepsy model.

References

Bezzi, P., Domercq, M., Brambilla, L., Galli, R., Schols, D., De Clercq, E., Vescovi, A., Bagetta, G., Kollias, G., Meldolesi, J., and Volterra, A., 2001. CXCR4-activated astrocyte glutamate release via TNFalpha: amplification by microglia triggers neurotoxicity. Nat Neurosci 4**,** 702-710.

Bovolenta, R., Zucchini, S., Paradiso, B., Rodi, D., Merigo, F., Navarro Mora, G., Osculati, F., Berto, E., Marconi, P., Marzola, A., Fabene, P. F., and Simonato, M., 2010. Hippocampal FGF-2 and BDNF overexpression attenuates epileptogenesis-associated neuroinflammation and reduces spontaneous recurrent seizures. J Neuroinflammation 7**,** 81.

Choi, J., Nordli, D. R., Jr., Alden, T. D., DiPatri, A., Jr., Laux, L., Kelley, K., Rosenow, J., Schuele, S. U., Rajaram, V., and Koh, S., 2009. Cellular injury and neuroinflammation in children with chronic intractable epilepsy. J Neuroinflammation 6**,** 38.

De Simoni, M. G., Perego, C., Ravizza, T., Moneta, D., Conti, M., Marchesi, F., De Luigi, A., Garattini, S., and Vezzani, A., 2000. Inflammatory cytokines and related genes are induced in the rat hippocampus by limbic status epilepticus. Eur J Neurosci 12**,** 2623-2633.

Fabene, P. F., Bramanti, P., and Constantin, G., 2010. The emerging role for chemokines in epilepsy. J Neuroimmunol 224**,** 22-27.

Frasca, A., Aalbers, M., Frigerio, F., Fiordaliso, F., Salio, M., Gobbi, M., Cagnotto, A., Gardoni, F., Battaglia, G. S., Hoogland, G., Di Luca, M., and Vezzani, A., 2011. Misplaced NMDA receptors in epileptogenesis contribute to excitotoxicity. Neurobiol Dis 43**,** 507-515.

Gardoni, F., Mauceri, D., Malinverno, M., Polli, F., Costa, C., Tozzi, A., Siliquini, S., Picconi, B., Cattabeni, F., Calabresi, P., and Di Luca, M., 2009. Decreased NR2B subunit synaptic levels cause impaired long-term potentiation but not long-term depression. J Neurosci 29**,** 669-677.

Hu, S., Sheng, W. S., Ehrlich, L. C., Peterson, P. K., and Chao, C. C., 2000. Cytokine effects on glutamate uptake by human astrocytes. Neuroimmunomodulation 7**,** 153-159.

Jarvela, J. T., Lopez-Picon, F. R., Plysjuk, A., Ruohonen, S., and Holopainen, I. E., 2011. Temporal profiles of age-dependent changes in cytokine mRNA expression and glial cell activation after status epilepticus in postnatal rat hippocampus. J Neuroinflammation 8**,** 29.

Kamikawa, H., Hori, T., Nakane, H., Aou, S., and Tashiro, N., 1998. IL-1beta increases norepinephrine level in rat frontal cortex: involvement of prostanoids, NO, and glutamate. Am J Physiol 275**,** R803-810.

Khurgel, M., and Ivy, G. O., 1996. Astrocytes in kindling: relevance to epileptogenesis. Epilepsy Res 26**,** 163-175.

Khurgel, M., Switzer, R. C., 3rd, Teskey, G. C., Spiller, A. E., Racine, R. J., and Ivy, G. O., 1995. Activation of astrocytes during epileptogenesis in the absence of neuronal degeneration. Neurobiol Dis 2**,** 23-35.

Lee, T. H., Lee, J. G., Yon, J. M., Oh, K. W., Baek, I. J., Nahm, S. S., Lee, B. J., Yun, Y. W., and Nam, S. Y., 2011. Capsaicin prevents kainic acid-induced epileptogenesis in mice. Neurochem Int 58**,** 634-640.

Lehtimaki, K., Peltola, J., Koskikallio, E., Keranen, T., and Honkaniemi, J., 2003. Expression of cytokines and cytokine receptors in the rat brain after kainic acid-induced seizures. Brain Res Mol Brain Res 110**,** 253-260.

Maroso, M., Balosso, S., Ravizza, T., Iori, V., Wright, C. I., French, J., and Vezzani, A., 2011. Interleukin-1beta biosynthesis inhibition reduces acute seizures and drug resistant chronic epileptic activity in mice. Neurotherapeutics 8**,** 304-315.

Maroso, M., Balosso, S., Ravizza, T., Liu, J., Bianchi, M. E., and Vezzani, A., 2011. Interleukin-1 type 1 receptor/Toll-like receptor signalling in epilepsy: the importance of IL-1beta and high-mobility group box 1. J Intern Med 270**,** 319-326.

Morimoto, K., Fahnestock, M., and Racine, R. J., 2004. Kindling and status epilepticus models of epilepsy: rewiring the brain. Prog Neurobiol 73**,** 1-60.

Moynagh, P. N., Williams, D. C., and O'Neill, L. A., 1993. Interleukin-1 activates transcription factor NF kappa B in glial cells. Biochem J 294 (Pt 2)**,** 343-347.

Nishio, S., Morioka, T., Hisada, K., and Fukui, M., 2000. Temporal lobe epilepsy: a clinicopathological study with special reference to temporal neocortical changes. Neurosurg Rev 23**,** 84-89.

Pernot, F., Heinrich, C., Barbier, L., Peinnequin, A., Carpentier, P., Dhote, F., Baille, V., Beaup, C., Depaulis, A., and Dorandeu, F., 2011. Inflammatory changes during epileptogenesis and spontaneous seizures in a mouse model of mesiotemporal lobe epilepsy. Epilepsia 52**,** 2315-2325.

Plata-Salaman, C. R., Ilyin, S. E., Turrin, N. P., Gayle, D., Flynn, M. C., Romanovitch, A. E., Kelly, M. E., Bureau, Y., Anisman, H., and McIntyre, D. C., 2000. Kindling modulates the IL-1beta system, TNF-alpha, TGF-beta1, and neuropeptide mRNAs in specific brain regions. Brain Res Mol Brain Res 75**,** 248-258.

Racine, R. J., 1972. Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol 32**,** 281-294.

Ravizza, T., Gagliardi, B., Noe, F., Boer, K., Aronica, E., and Vezzani, A., 2008. Innate and adaptive immunity during epileptogenesis and spontaneous seizures: evidence from experimental models and human temporal lobe epilepsy. Neurobiol Dis 29**,** 142-160.

Ravizza, T., Noe, F., Zardoni, D., Vaghi, V., Sifringer, M., and Vezzani, A., 2008. Interleukin Converting Enzyme inhibition impairs kindling epileptogenesis in rats by blocking astrocytic IL-1beta production. Neurobiol Dis 31**,** 327-333.

Ravizza, T., Rizzi, M., Perego, C., Richichi, C., Veliskova, J., Moshe, S. L., De Simoni, M. G., and Vezzani, A., 2005. Inflammatory response and glia activation in developing rat hippocampus after status epilepticus. Epilepsia 46 Suppl 5**,** 113-117.

Rijkers, K., Aalbers, M., Hoogland, G., van Winden, L., Vles, J., Steinbusch, H., and Majoie, M., 2010. Acute seizure-suppressing effect of vagus nerve stimulation in the amygdala kindled rat. Brain Res 1319C**,** 155-163.

Rosell, D. R., Nacher, J., Akama, K. T., and McEwen, B. S., 2003. Spatiotemporal distribution of gp130 cytokines and their receptors after status epilepticus: comparison with neuronal degeneration and microglial activation. Neuroscience 122**,** 329-348.

Ryu, H. J., Kim, J. E., Kim, M. J., Kwon, H. J., Suh, S. W., Song, H. K., and Kang, T. C., 2010. The protective effects of interleukin-18 and interferon-gamma on neuronal damages in the rat hippocampus following status epilepticus. Neuroscience 170**,** 711-721.

Sander, J. W., 2003. The epidemiology of epilepsy revisited. Curr Opin Neurol 16**,** 165-170.

Sayyah, M., Beheshti, S., Shokrgozar, M. A., Eslami-far, A., Deljoo, Z., Khabiri, A. R., and Haeri Rohani, A., 2005. Antiepileptogenic and anticonvulsant activity of interleukin-1 beta in amygdala-kindled rats. Exp Neurol 191**,** 145-153.

Shandra, A. A., Godlevsky, L. S., Vastyanov, R. S., Oleinik, A. A., Konovalenko, V. L., Rapoport, E. N., and Korobka, N. N., 2002. The role of TNF-alpha in amygdala kindled rats. Neurosci Res 42**,** 147-153.

Tooyama, I., Bellier, J. P., Park, M., Minnasch, P., Uemura, S., Hisano, T., Iwami, M., Aimi, Y., Yasuhara, O., and Kimura, H., 2002. Morphologic study of neuronal death, glial activation, and progenitor cell division in the hippocampus of rat models of epilepsy. Epilepsia 43 Suppl 9**,** 39-43.

Tuunanen, J., and Pitkanen, A., 2000. Do seizures cause neuronal damage in rat amygdala kindling? Epilepsy Res 39**,** 171-176.

Vezzani, A., French, J., Bartfai, T., and Baram, T. Z., 2011. The role of inflammation in epilepsy. Nat Rev Neurol 7**,** 31-40.

Vezzani, A., Moneta, D., Conti, M., Richichi, C., Ravizza, T., De Luigi, A., De Simoni, M. G., Sperk, G., Andell-Jonsson, S., Lundkvist, J., Iverfeldt, K., and Bartfai, T., 2000. Powerful anticonvulsant action of IL-1 receptor antagonist on intracerebral injection and astrocytic overexpression in mice. Proc Natl Acad Sci U S A 97**,** 11534-11539.

Vezzani, A., Moneta, D., Richichi, C., Aliprandi, M., Burrows, S. J., Ravizza, T., Perego, C., and De Simoni, M. G., 2002. Functional role of inflammatory cytokines and antiinflammatory molecules in seizures and epileptogenesis. Epilepsia 43 Suppl 5**,** 30-35.

Voutsinos-Porche, B., Koning, E., Kaplan, H., Ferrandon, A., Guenounou, M., Nehlig, A., and Motte, J., 2004. Temporal patterns of the cerebral inflammatory response in the rat lithium-pilocarpine model of temporal lobe epilepsy. Neurobiol Dis 17**,** 385-402.

Wood, J. C., Jackson, J. S., Jakubs, K., Chapman, K. Z., Ekdahl, C. T., Kokaia, Z., Kokaia, M., and Lindvall, O., 2011. Functional integration of new hippocampal neurons following insults to the adult brain is determined by characteristics of pathological environment. Exp Neurol 229**,** 484-493.

Yi, P. L., Tsai, C. H., Lin, J. G., Lee, C. C., and Chang, F. C., 2004. Kindling stimuli delivered at different times in the sleep-wake cycle. Sleep 27**,** 203-212.

Chapter 5

Misplaced NMDA receptors in epileptogenesis contribute to excitotoxicity

A. Frasca, M. Aalbers, F. Frigerio, F. Fiordaliso, M. Salio, M. Gobbi, A. Cagnotto, F. Gardoni, G. Battaglia, G. Hoogland, M. Di Luca, A. Vezzani

Neurobiology of Disease 2011;43:507-15

Abstract

Pharmacological blockade of NR2B-containing N-Methyl-D-Aspartate receptors (NMDARs) during epileptogenesis reduces neurodegeneration provoked in the rodent hippocampus by status epilepticus. The functional consequences of NMDAR activation are crucially influenced by their synaptic versus extra-synaptic localization, and both NMDAR function and localization are dependent on the presence of the NR2B subunit and its phosphorylation state. We investigated whether changes in NR2B subunit phosphorylation, and alterations in its neuronal membrane localization and cellular expression occur during epileptogenesis, and if these changes are involved in neuronal cell loss. We also explored NR2B subunit changes in the acute phase of status epilepticus and in the chronic phase of spontaneous seizures, which encompass the epileptic process.

Levels of Tyr^{1472} phosphorylated NR2B subunit decreased in the post-synaptic membranes from rat hippocampus during epileptogenesis induced by electrical status epilepticus. This effect was concomitant with reduced interactions between NR2B and post-synaptic density (PSD)-95 protein and was associated with decreased CREB phosphorylation. This evidence suggests an extra-synaptic localization of the NR2B subunit in epileptogenesis. Accordingly, electron microscopy showed increased NR2B both in extra-synaptic and pre-synaptic neuronal compartments, and a concomitant decrease of this subunit in post-synaptic densities (PSD) indicating a shift in NR2B membrane localization. *De novo* expression of NR2B in activated astrocytes was also found in epileptogenesis indicating ectopic receptor expression in glia. NR2B phosphorylation changes at completion of status epilepticus and interictally in the chronic phase of spontaneous seizures are predictive of receptor translocation to extra-synaptic sites.

Pharmacological blockade of NR2B-containing NMDARs by ifenprodil administration during epileptogenesis significantly reduced pyramidal cell loss in the hippocampus, showing that the observed post-translational and cellular changes of NR2B subunit contribute to excitotoxicity. Therefore, targeting misplaced NR2B-containing NMDARs, or preventing these NMDAR changes, should be considered to block excitotoxicity, which develops after various epileptogenic brain injuries.

Introduction

N-methyl-D-aspartate receptors (NMDAR) play a key role in synaptic transmission, long-term potentiation (Bliss and Collingridge, 1993), excitotoxic neuronal damage (Choi and Rothman, 1990, Mody and MacDonald, 1995), and seizures (Dingledine, et al., 1990). NMDARs are heterotetrameric complexes of two constitutive glycine-binding NR1 subunits combined with two regulatory glutamate-binding NR2 subunits (i.e. A, B, C, D). NR3 subunits can assemble with NR1 and NR2 subunits to decrease NMDAR current amplitudes, or with the NR1 subunit alone to form glycine-activated receptors (Chatterton, et al., 2002, Dingledine, et al., 1999).

The presence of the NR2B subunit critically influences not only the pharmacological and electrophysiological properties of the NMDAR but also its cellular membrane distribution (Dingledine, et al., 1999). NMDARs are mainly localized at the post-synaptic densities (PSD), where they are anchored to scaffolding proteins (e.g. PSD-95, SAP-102, SAP-93), while NMDARs containing the NR2B subunit have also been identified extrasynaptically (Petralia, et al., 2010, Tovar and Westbrook, 2002) or presynaptically (Jourdain, et al., 2007, Woodhall, et al., 2001). The localization of NMDARs is an important factor that determines the functional consequences of receptor activation. For instance, *post-synaptic NMDARs* are activated by synaptically-released glutamate and mediate long-term potentiation and long-term depression of synaptic transmission (Bear and Malenka, 1994). The activation of these receptors results in CREB-dependent transcription of genes which are responsible for neuroprotection against different types of insults (e.g. apoptotic, excitotoxic, necrotic or oxidative) (Papadia and Hardingham, 2007). However, NMDAR over-activation can mediate excitotoxic effects due to excessive neuronal Ca^{2+} influx (Forder and Tymianski, 2009). Conversely, *extra-synaptic NR2B-containing NMDARs* are predominantly activated by glutamate released by astrocytes (Jourdain, et al., 2007) or spilled over from the synaptic cleft during episodes of high frequency synaptic activity (Conti and Weinberg, 1999). Activation of these receptors causes CREB dephosphorylation and contributes to the mechanisms of neuronal cell death (Fellin, et al., 2004, Papadia and Hardingham, 2007). In the hippocampus, *pre-synaptic NMDARs* have been described and reported to promote glutamate release, thus augmenting the concentration of extracellular glutamate (Langer, 2008, Martin, et al., 1991).

In addition to composition and localization, the phosphorylation of NMDAR subunits by the Src tyrosine kinase family is a key factor for determining receptor function. Thus, Tyr^{1472} phosphorylation of NR2B by Src kinases leads to up-regulation of NMDAR function by increasing channel permeability to Ca^{2+} (Ali and Salter, 2001) and stabilizing the receptor at the PSD (Collingridge, et al., 2004, Salter and Kalia, 2004).

It was demonstrated that Ca^{2+} overload via activated post-synaptic, as well as extra-synaptic and pre-synaptic NMDARs, contributes to neuronal hyperexcitability (Kohl and Dannhardt, 2001, Rice and DeLorenzo, 1998) and excitotoxicity in seizure models

(Araujo, et al., 2008, Fellin, et al., 2004, Sierra-Paredes and Sierra-Marcuno, 2007, Yang, et al., 2006). Pre-synaptic NR2B-containing NMDARs facilitate glutamate release in the entorhinal cortex of epileptic rats (Yang, et al., 2006), thus promoting excitotoxicity and reinforcing seizures via an increase in glutamatergic neurotransmission. Increased NR2B subunit phosphorylation was reported in post-synaptic membranes of rat forebrain during the first 24 h after the onset of status epilepticus (Huo, et al., 2006, Moussa, et al., 2001, Niimura, et al., 2005). In addition, a decrease of NR1 and NR2B levels have been reported in human neocortical epilepsy specimens (Auzmendi, et al., 2009, Sun, et al., 2009, Wyneken, et al., 2003) and in cortical post-synaptic membranes or hippocampal homogenates in rats with either provoked (Auzmendi et al., 2009) or spontaneous seizures (Auzmendi, et al., 2009, Sun, et al., 2009, Wyneken, et al., 2003). Decreased NR2B mRNA levels were also reported in pyramidal neurons of temporal lobe epilepsy patients with hippocampal sclerosis (Mathern et al., 1998). Conversely, an upregulation of NR2B mRNA was found in pyramidal cells of non sclerotic hippocampi from epileptic patients (Mathern et al., 1998) and NR2B levels are increased in post-synaptic membranes in epileptic foci from focal cortical dysplasia (Mikuni et al., 1999; Colciaghi et al, 2011).

Although some information exists on NR2B expression and phosphorylation in the acute and chronic phases of seizures, no studies are available regarding NR2B subunit levels, its phosphorylation state and membrane localization during epileptogenesis. This knowledge is crucial to understand the role of NMDAR in this post-injury period prodromal to epilepsy development. The focus of our study was therefore to obtain this information by applying a multidisciplinary approach to the epileptogenesis phase preceding spontaneous seizures in a rat model of TLE, one of the most common and drug-resistant forms of human epilepsy (Majores, et al., 2007).

We also examined NR2B subunit levels and its phosphorylation in the acute phases of status epilepticus and after spontaneous seizures to provide information in the same epilepsy model on the dynamic changes in NR2B anticipated by the scattered information in the literature. These results are presented in the Supplementary Material.

Our data show that the NR2B subunit is increased in neurons in pre-synaptic and extra-synaptic compartments during epileptogenesis, in concomitance with decreased NR2B post-synaptic localization and phosphorylation. Moreover, ectopic expression of both NR2B and NR1 occurred in activated astrocytes. Changes in NR2B during status epilepticus and spontaneous seizures are predictive of receptor translocation to extra-synaptic sites. Blockade of NR2B-containing NMDAR with ifenprodil during epileptogenesis significantly reduced neuronal cell loss.

Our findings indicate that therapeutic post-injury interventions targeting misplaced NMDARs could afford neuroprotection while limiting interference with physiological neurotransmission mediated by synaptic NMDARs.

Materials and methods

Experimental animals

Adult male Sprague-Dawley rats (225-250 g) were purchased from Charles River (Calco, Italy) and were housed at constant temperature (23°C) and relative humidity (60 ± 5%) with free access to food and water and a fixed 12 h light/dark cycle. All experimental procedures were conducted in conformity with institutional guidelines that are in compliance with national (D.L.n.116, G.U., Suppl 40, February 18, 1992) and international guidelines and laws (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987, Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

Self-sustained limbic status epilepticus (SSLSE)

A total number of 104 rats were used to induce SSLSE. Five rats were excluded from this study since they did not develop status epilepticus. Rats were stereotactically implanted under Equithesin anaesthesia with cortical and hippocampal electrodes as previously described in detail (Ravizza, et al., 2008). Rats were allowed to recover from the surgical procedures for 7 days. Then they were unilaterally stimulated (50 Hz, 400 μA peak-to-peak, 1ms biphasic square waves in 10 s trains delivered every 11 s, i.e. 10 s on, 1 s off) in the CA3 region of the ventral hippocampus for 60 min to induce SSLSE according to a previously established protocol (De Simoni, et al., 2000, Noe, et al., 2008). EEG was recorded in each freely-moving rat for ~30 min to establish a pre-stimulation baseline, then every 10 min for 1 min in the absence of electrical stimulation, i.e. the "stimulus-off" period. All rats selected for subsequent analysis showed an EEG pattern of uninterrupted bilateral spikes in the hippocampi during the "stimulus-off" period, starting between the first and the fourth epoch of stimulation onwards. These criteria selected rats developing SSLSE, which remitted within 24 h from the initial stimulation (De Simoni, et al., 2000, Noe, et al., 2008).

EEG recordings after SSLSE

After SSLSE induction, continuous EEG monitoring was done for 96 h and then recording was interrupted and resumed after 1 month for 2 consecutive weeks (24 h/day, 7 days/week) to detect spontaneous recurrent seizures (SRS). In accordance with previous findings (Ravizza, et al., 2008), EEG analysis determined sequential temporal phases of the epileptic process after SSLSE onset: the *acute phase* of self-sustained epileptic activity exemplified by choosing two time points, i.e. 2 and 18 h (*Supplementary Data*); the *epileptogenic phase* (96 h from SE onset) encompassing the period devoid of seizure activity, from the end of SE until the onset of spontaneous seizures; the *chronic phase of spontaneous recurrent seizures (SRS),* 1 month after

SSLSE induction (Supplementary Data). Two independent investigators analyzed the EEG visually off-line. Control rats were implanted with electrodes but not stimulated.
Distinct groups of rats were used for the various experiments as reported in Supplementary Table S5.1.

Ifenprodil treatment

Ifenprodil (tartrate salt, gift from Sanofi-Aventis, Bagneux, France), an antagonist of NR2B-containing NMDARs (Williams, 2001), was dissolved in distilled water and injected 20 mg/kg, i.p. 24 h after SSLSE onset, then once daily for 3 additional days (n=8). Control sham-implanted animals were similarly injected with vehicle (n=8) or ifenprodil (n=4).

Immunohistochemistry

Rats (n=4-8) were deeply anaesthetized with Equithesin and transcardially perfused with phosphate buffered saline (50 mM PBS; pH 7.4) followed by chilled 4% paraformaldehyde in PBS. The brains were prepared for immunohistochemistry as previously described (Ravizza, et al., 2008). Serial cryostat horizontal sections (40 µm) were cut throughout the temporal extension of the hippocampus (from plate 56 to plate 62) (Paxinos and Watson, 1986), then collected in 100 mM PBS for immunohistochemical analysis as detailed below.

Double-immunostaining

To identify the cells expressing NR2B, 3 slices were used in each rat brain for each cell marker, namely plate 56, 59, and 62 (Paxinos and Watson, 1986). Freely-floating slices were incubated at 4°C for 30 min in 0.5% H_2O_2 in Tris-HCl-buffered saline (TBS), followed by 60-min incubation in 10% bovine serum albumin (BSA) in 10% fetal calf serum (FCS) in TBS. The slices were then incubated overnight at 4°C in 10% BSA in 10% FCS in TBS with anti-NR2B (1:200, Zymed Laboratories, San Francisco, CA) rabbit polyclonal antibody, then in anti-rabbit secondary antibody conjugated with Alexa 488 (Molecular Probes, Leiden, The Netherlands). Sections were subsequently incubated with the following primary antibodies: mouse anti-glial fibrillary acidic protein (GFAP, 1:4000, Chemicon Int. Inc. Temecula, USA) as a marker of astrocytes or mouse anti-CD11b (complement receptor type 3, OX-42, 1:100, Serotec Ltd, Oxford, UK) as a marker of microglia/macrophages, or with mouse anti-neuronal specific nuclear protein (NeuN, 1:1000, Chemicon), a selective neuronal marker, as previously described (Ravizza, et al., 2008). Fluorescence was detected using anti-mouse secondary antibody conjugated with Alexa 546 (Molecular Probes).
To assess the presence of functional NMDARs in astrocytes, we determined the expression of the constitutive NR1 subunit. Two rats were randomly chosen from each experimental group and 2 slices from each animal were incubated with rabbit anti-NR1

polyclonal antibody (1:50, Chemicon), then in anti-rabbit secondary antibody conjugated with Alexa 488 (Molecular Probes). Sections were subsequently stained with mouse anti-GFAP monoclonal antibody and fluorescence was detected using anti-mouse secondary antibody conjugated with Alexa 546 (Molecular Probes). Slide-mounted sections were examined with an Olympus Fluorview laser scanning confocal microscope (microscope BX61 and confocal system FV500; Hamburg, Germany) using dual excitation of 488 nm (Laser Ar) and 546 nm (Laser He–Ne green) for Fluorescein and Alexa 546, respectively. The emission of fluorescent probes was collected on separate detectors. To eliminate the possibility of bleed-through between channels, the sections were scanned in a sequential mode.

Neuronal cell injury/death

Cell loss and ongoing neurodegeneration were evaluated using NeuN-stained and Fluoro-Jade A (FJA) slices, respectively. We prepared 8 series of 9 sections each, encompassing the temporal aspect of the hippocampus from plate 56 to plate 62 (Paxinos and Watson, 1986). In each series, the first and second sections were stained for FJA and NeuN, respectively. FJA labeling was carried out as previously described (Ravizza and Vezzani, 2006). For NeuN staining, sections were incubated with mouse anti-NeuN (1:1000, Chemicon) (Ravizza, et al., 2008), then with anti-mouse secondary antibody conjugated with Alexa 546. Finally, sections were counterstained with Hoechst 33258 (1:500, Molecular Probes) to visualize the cell nucleus, then coverslipped with FluorSave (Calbiochem, San Diego, CA).

Cell Counting

Cell counting was performed in 8 slices in each rat brain stained with FJA and NeuN-Hoechst by quantifying the number of CA1 and CA3 pyramidal cells and the hilar interneurons in the stimulated hippocampi (n=4-8 rats in each group), as previously described (Ding, et al., 2007). For the quantification of hilar interneurons, 2 adjacent non-overlapping fields at 20X (700 μm x 475 μm each field) were selected both in NeuN-Hoechst and FJA-labeled sections; CA1 and CA3 pyramidal cells were counted in one field at 20X (700 μm x 475 μm) for both neuronal markers. Images were captured and digitized using an Olympus Fluorview laser scanning confocal microscope with excitation of 488 nm (Ar Laser) for FJA staining and dual excitation of 546 nm (He–Ne Laser green) and 350 nm (ultraviolet) for NeuN-Hoechst labeled neurons. We considered only pyramidal cells and hilar interneurons where the NeuN staining was clearly associated with Hoechst signal, while we counted all FJA-positive cells. Cells matching the above criteria of inclusion were identified by two independent investigators blind to the treatments, and an automated cell count was generated using ImageJ software. Then, we measured the hilar area and the area occupied by pyramidal cells in CA1 and CA3 (in μm^2) in each field using ImageJ. For each hippocampal subfield (CA1, CA3, and hilus) in each slice, the number of counted cells

was divided by the area, thus providing a value of cellular density (number of cells/mm^2). Data obtained in each slice were averaged providing a single value for each rat, and this value was used for the statistical analysis. Although this cell counting method has some limitations as compared to designed-based stereological analysis (Schmitz and Hof, 2005), the occurrence of any bias in counting should similarly affect sham and experimental samples since these samples underwent the same procedure in parallel.

Immuno-electron microscopy

Experimental rats and their controls (n=2) were deeply anaesthetized with Equithesin and transcardially perfused with 50 mM PBS (pH 7.4) followed by chilled 2% paraformaldehyde and 1% glutaraldehyde in PBS for 5 min. Stratum radiatum of hippocampal CA3, the region with highest amount of synaptic contacts, was excised from each rat brain using a razor blade to obtain specimens suitable to allow orientation and proper fixative penetration (2% paraformaldehyde and 1% glutaraldehyde for 2 h at room temperature). Samples were then transferred in test tubes and embedded sequentially at 37° C in 2%, 5%, and 12% gelatin for 30 min each step (Sigma). Tubes were put on ice until gelatin solidification. The embedded samples were cut in 0.5-1 mm^3 blocks and placed in 2.3 M sucrose in PBS overnight in a rotating wheel at 4°C. After removing the excess sucrose, tissue samples were placed on holders and immediately frozen in liquid nitrogen. The holder with the annexed tissue block was mounted to the arm of a Leica EM UC6 ultramicrotome equipped with a cryochamber (Leica EM UC6) and the sample was trimmed and then sectioned at 50 nm thickness. Three ultrathin adjacent sections from each brain specimen were collected on formvar-coated copper grids and incubated with anti-NR2B rabbit polyclonal antibody (1:50, Zymed Laboratories) in 1% BSA in PBS overnight at 4°C, followed by a protein A-gold (10 nm) complex for 30 min (Cell Microscopy Center, University Medical Center Utrecht). Grids were counterstained with 0.4% uranyl acetate and examined with an Energy Filter Transmission Electron Microscope (EFTEM, ZEISS LIBRA® 120) equipped with a YAG scintillator slow scan CCD camera. Symmetric and asymmetric synapses were identified as described previously (Colonnier, 1968).
In each experimental group, quantification of immunolabeling of NR2B in CA3 stratum radiatum was done as follows: 1. The density of gold particles labeling NR2B was assessed by dividing the total number of particles close to the synaptic contacts by the stratum radiatum CA3 area (in μm^2) as measured by iTem software (Olympus Soft Imaging Solutions, Germany); 2. The number of gold particles in 4 different areas as defined below was expressed as percentage of total NR2B immunolabeling in stratum radiatum CA3. The 4 areas studied were the pre-synaptic membrane defined by the presence of synaptic vesicles, the post-synaptic membrane defined by the zone within 100 nm from the PSD, the peri-synaptic zone (100-300 nm from the PSD) and the extra-synaptic zone (>300 nm from the PSD) (Groc, et al., 2009, Petralia, et al., 2010).

Western blot

Rats (n=4-6 in each experimental group) were decapitated and ventral hippocampi of both hemispheres were dissected out, frozen on dry ice and stored at -80°C. Total protein content was measured in the homogenate or subcellular fractions by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Milano, Italy).

Stimulated hippocampi were homogenized (40 mg/ml) and used for subcellular fractionation as previously described (Gardoni, et al., 2009). Control rats were implanted with electrodes but not stimulated (sham group, n=14): 4 rats were matched with 2 and 18 h exp rats and 4 rats with the chronic phase rats (Supplementary Figure S5.1); 6 rats were matched with the 96 h exp rats (Figure 5.1).

The *post-synaptic density (PSD)-enriched fraction* was used to measure P-NR2B, NR2B, NR1 and PSD-95. Gels (8% SDS-PAGE) were run under reducing conditions for each protein; 10 µg proteins from each sample were run in duplicate and membranes obtained from electroblotting were probed using the following antibodies: anti-pTyr^{1472}-NR2B rabbit polyclonal antibody (1:500; Thermo Scientific, Waltham, MA); anti-NR2B rabbit polyclonal antibody (1:1000; Zymed Laboratories); anti-NR1 (1:1000; Chemicon) and anti-PSD-95 (1:2000; Cayman Chemical, Ann Arbor, MI) mouse monoclonal antibodies. Blots used to measure pTyr^{1472}-NR2B were stripped, then re-probed to measure total levels of NR2B as previously described (Fumagalli, et al., 2008).

CREB in homogenate: Hippocampi contralateral to the stimulated side were homogenized (40 mg/750 µl) as described previously (Pozzi, et al., 2003). Thirty µg proteins were run in duplicate on 11% SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories), then incubated with anti-pSer^{133}-CREB monoclonal antibody (1:4000; Upstate, Temecula, CA). To measure total levels of CREB, the same blots were stripped and incubated with anti-CREB polyclonal antibody (1:4000; Upstate).

Immunoreactivity was visualized with enhanced chemiluminescence (ECL, Amersham, UK) using peroxidase-conjugated goat anti-rabbit (1:2000; Sigma) or rabbit anti-mouse (1:2000; Sigma) IgGs as secondary antibodies. Densitometric analysis of immunoblots was done by Quantity One software (Bio-Rad Laboratories) to quantify the changes in protein levels using film exposures with maximal signals below the photographic saturation point. Optical density values in each sample were normalized using the corresponding amount of β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoprecipitation

To assess the fraction of NR2B associated to PDS-95, immunoprecipitation was carried out as previously described (n= 3 rats in each experimental group) (Gardoni, et al., 2006). Briefly, aliquots of hippocampal homogenates (100 µg) from stimulated hippocampi were incubated overnight at 4°C in buffer A (200 mM NaCl, 10 mM EDTA, 10 mM Na_2HPO_4, 0.5% NP-40 and 0.1% SDS) containing anti-PSD-95 mouse monoclonal antibody (1:100; Neuromab, Davis, CA). Protein A-agarose beads (Santa Cruz

Biotechnology) washed in buffer A, were added and incubation was continued for 2 h at 4°C. The beads were collected by centrifugation and washed 5 times with buffer A. Sample buffer for SDS-PAGE was added and the mixture was boiled for 10 min. Beads were pelleted by centrifugation and the supernatants were applied to 7% SDS-PAGE.

Ifenprodil binding

A total number of 36 rats were used for this analysis. Three independent experiments were run using a total of 12 rats per experiment, i.e. 6 rats in each experimental group. Hippocampi obtained from each group of 6 rats were pooled to yield enough proteins in the P3 and PSD fractions. This procedure produced 3 independent final values in each experimental group for statistical analysis of data.
[^{3}H]-Ifenprodil binding to NR2B-containing NMDAR was carried out as previously described (Grimwood, et al., 2000) using the total membrane fraction (P3) and the PSD-enriched fraction from subcellular fractionation of stimulated or sham hippocampi. Briefly, the pellets were resuspended in 50 mM Tris-acetate (pH 7.0) containing 100 µM (+)-3PPP (a blocker of sigma receptors) and 1 µM GBR 12909 (a blocker of dopamine transporter), to a final concentration of 20 µg/µl original tissue: 300 µl were incubated for 2 h at 4°C with 8 nM [^{3}H]-Ifenprodil (40 Ci/mmol, Perkin Elmer) in the absence or presence of different concentrations (from 10^{-10} M to 10^{-5} M) of unlabelled ifenprodil (homologous competition). Samples were then filtered through Whatman GF/B filters (pre-soaked in ice-cold assay buffer containing 0.05% polyethylenimine) using a Brandell cell harvester. Filters were soaked overnight in 5 ml of liquid scintillation Ultima Gold MV (Packard) and finally counted in a β-counter (Tri-Carb 2800 TR, Beckman).
Inhibition curves were fitted using the “one site homologous competition” equation (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com) for the estimation of the affinity (Kd) of ifenprodil binding and the number of NR2B-containing NMDARs (Bmax). The Bmax measured in the PSD-enriched fraction was calculated as percentage of the Bmax measured in the total P3 fraction (from which PSD-fraction was obtained).

Statistical analysis

Data are presented as mean ± SEM (number of individual samples or rats). For western blot analysis, the mean value of the control group was set at 100 and the single values from experimental rats were expressed as percentage of corresponding mean control value. Statistical analysis of changes in the phosphorylation or protein level was performed using one-way ANOVA followed by Dunnett’s test. For cell counting, Student’s t-test was used for FJA-positive cells while two-way ANOVA followed by Tukey’s test was used for NeuN-Hoechst labeled cells. Data from the binding experiment were analyzed using Mann-Withney test. Data from immuno-electron

microscopy were analyzed using Student's t-test. Differences due to the treatments were considered significant with $p<0.05$.

Results

The level and phosphorylation of the NR2B subunit are decreased in PSD during epileptogenesis

The hippocampal levels of Tyr^{1472} phosphorylated NR2B subunit (P-NR2B) were decreased in PSD-enriched fraction by 37 ± 13% below control values 96 h after SE onset ($p<0.05$); at the same time, the total levels of NR2B, NR1, and PSD-95 were significantly reduced by 25 to 44% ($p<0.05$ and $p<0.01$) as assessed by western blot (Figure 5.1A).

Supplementary Figure S5.1A depicts the changes in P-NR2B during the acute phases of seizures and in chronic epileptic rats: 2 h after SE onset or 2 h after the occurrence of a spontaneous seizure, P-NR2B levels were significantly increased over control values by 50 ± 23% ($p<0.01$) and by 40 ± 13% ($p<0.05$), respectively. This increase was transient, and reverted to a significant decrease 24 h after a spontaneous seizure (-62 ± 9%, $p<0.01$). The total levels of NR2B, NR1, and PSD-95 were reduced below control values similarly to epileptogenesis (Supplementary Figure S5.1A,B).

Extra-synaptic and pre-synaptic localization of NMDAR during epileptogenesis

To determine the specific membrane localization of the NR2B subunit during epileptogenesis, we used several complementary approaches. First, we analyzed Ser^{133} phosphorylation of CREB (Figure 5.1B) and we found a reduction in CREB phosphorylation (-53 ± 10%, $p<0.05$), thus supporting the activation of extra-synaptic NMDARs (Hardingham and Bading, 2002). Second, co-immunoprecipitation experiments showed a drastic decrease in NR2B subunit associated with the synaptic protein PSD-95 during epileptogenesis (Figure 5.1C), thus supporting a reduction of NR2B in the post-synaptic compartment.

Third, the total number of receptors, as reflected by the Bmax of [^{3}H]-Ifenprodil binding, was measured both in the P3 fraction (total membrane preparation) and in the PSD-enriched fraction (the Triton X100-insoluble component of P3). No binding was detectable in the Triton X100-soluble fraction, likely because of Triton interference with the binding. During epileptogenesis, NR2B receptors in PSD-enriched membranes (Bmax 9.0 ± 4.0 fmol/ww tissue; n=3 independent experiments from 6 rats in each experimental group) were reduced to 38 ± 5% ($p<0.05$) over the total [^{3}H]-Ifenprodil P3 binding (Bmax 24.0 ±10.0 fmol/ww tissue) as compared to 62 ± 6% NR2B receptors in PSD fraction of sham rats (Bmax 22.0 ± 8.0 fmol/ww tissue;) over the total [^{3}H]-

Ifenprodil P3 binding (Bmax 34.0 ± 9.0 fmol/mg ww tissue). The Bmax value in the P3 fraction during epileptogenesis (Bmax 24.0 ±10.0 fmol/ww tissue) was decreased by 29 ± 14% as compared to sham rats (Bmax 34.0 ± 9.0 fmol/mg ww tissue), although not significantly. The affinity of [^{3}H]-Ifenprodil for NR2B-containing receptors in epileptogenesis (PSD, Kd, 27 ± 11; P3, Kd, 82 ± 21 nM) was similar to sham rats (PSD, Kd, 36 ± 12; P3, Kd, 75 ± 12 nM).

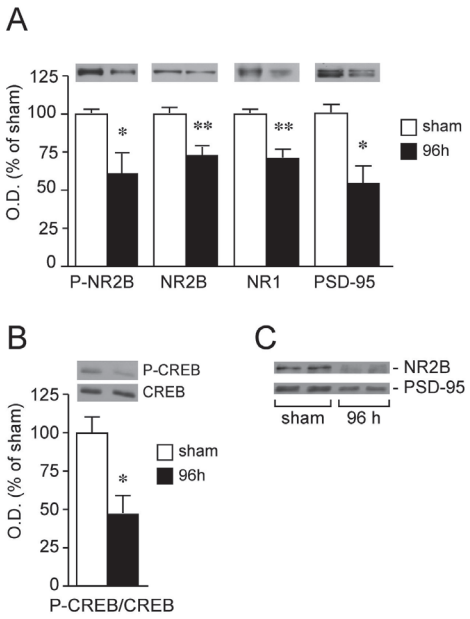


Figure 5.1 Changes in NR2B, NR1, and PSD-95 and biochemical evidence of extra-synaptic NR2B-containing NMDAR during epileptogenesis.

Bargrams in A and B show mean ± SEM (see Supplementary Table S5.1) of the optical density (O.D) values of relevant bands divided by the corresponding β-actin (internal standard). Data are expressed as percentage of control values (sham rats). Representative bands of each protein are depicted upon the respective bargrams.

A. Western blot analysis of Tyr^{1472} phosphorylated NR2B (P-NR2B), total NR2B, NR1, and PSD-95 protein levels in the PSD-enriched fraction of the stimulated hippocampus during epileptogenesis (n=6) (i.e. 96 h from status epilepticus onset). *p<0.05; **p<0.01 by one-way ANOVA followed by Dunnett's test (statistical analysis included also time points depicted in Supplementary Figure S5.1A,B; Sham rats=14).

B. Western blot analysis of CREB phosphorylation in the homogenates prepared from hippocampi contralateral to the stimulation site (n=6/group). CREB phosphorylation was measured by the ratio between the Ser^{133} phosphorylated and the total protein level (P-CREB/CREB). *p<0.05 by Student's t-test.

Panel C. Co-immunoprecipitation of NR2B and PSD-95 in hippocampal homogenates from sham rats (n=3) and during epileptogenesis (96 h) (n=3). The blot depicts the relevant bands; NR2B was associated with PSD-95 only in sham rats but not in experimental rats.

The specific NR2B membrane localization was investigated by immuno-electron microscopy: Figure 5.2A-D shows a different NR2B distribution in the hippocampus (i.e. stratum radiatum CA3) during epileptogenesis vs sham rats. In sham rats, ~50% of NR2B labeling was associated with PSD (Figure 5.2A,D) while the remaining labeling was predominantly distributed at peri-synaptic and extra-synaptic sites; a minor component was measured pre-synaptically. During epileptogenesis, NR2B labeling was significantly reduced by 51% ($p<0.05$) in PSD, while it was significantly increased both in the pre- and extra-synaptic compartments ($p<0.05$, Figure 5.2B-D). The majority of synapses in the analyzed area were asymmetric, i.e. 78.5% and 70.2% in sham and stimulated rats, respectively. The total number of NR2B was decreased during epileptogenesis (435 ± 31 gold particles/mm^2, $p<0.05$) as compared to sham rats (638 ± 65 gold particles/mm^2).

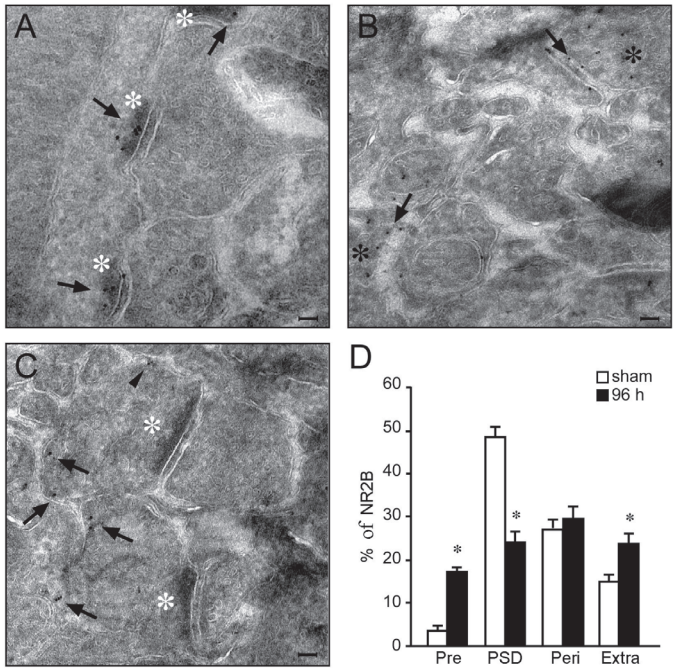


Figure 5.2 Membrane distribution of NR2B subunits in synapses of CA3 stratum radiatum during epileptogenesis as assessed by electron-microscopy.
Panel A depicts a representative image of stratum radiatum CA3 of a sham rat showing the immunogold labeling of NR2B (arrows) strictly associated with post-synaptic densities (PSD) (white asterisks). Panels B,C depict a representative comparable hippocampal section from a rat killed during epileptogenesis (i.e. 96 h from SE onset). Panel B shows NR2B subunit localized in pre-synaptic terminals (*Pre*) (arrows denote membrane associated immunogold labeling; black asterisks mark synaptic vesicles); panel C shows NR2B in peri-synaptic (*Peri*) (arrowhead denotes membrane associated immunolabeling) and in extra-synaptic (*Extra*) compartments (arrows denote membrane associated immunolabeling). See Methods for details. Scale bar: 100 nm. Bargrams in panel D show the distribution of NR2B immunolabeling in sham rats (white) and in rats during epileptogenesis (black).

Ectopic localization of NR2B in activated astrocytes during epileptogenesis

We analyzed the cellular expression of NR2B subunit by studying its co-localization with specific neuronal and glial cell markers.

NR2B were upregulated in GFAP-positive astrocytes (Figure 5.3G) but not in microglia (Figure 5.3B,F) during epileptogenesis while a specific neuronal expression was observed in control hippocampal sections (Figure 5.3A). Moreover, during epileptogenesis the number of NR2B-positive neurons was decreased (Figure 5.3E vs. A) likely reflecting neuronal cell loss (see Figure 5.4). Similarly to NR2B, increased astrocytic expression of NR1 was found in the same brain sections during epileptogenesis (Figure 5.3H) while only neuronal expression was detected in control sections (Figure 5.3D). Chronic epileptic rats showed changes in NR2B and NR1 similar to epileptogenesis while the pattern of receptor expression 2 h and 18 h after status epilepticus was similar to control hippocampi (not shown).

The increased NR2B and NR1 staining in astrocytes indicate that functional receptors are ectopically expressed in activated glial cells during epileptogenesis.

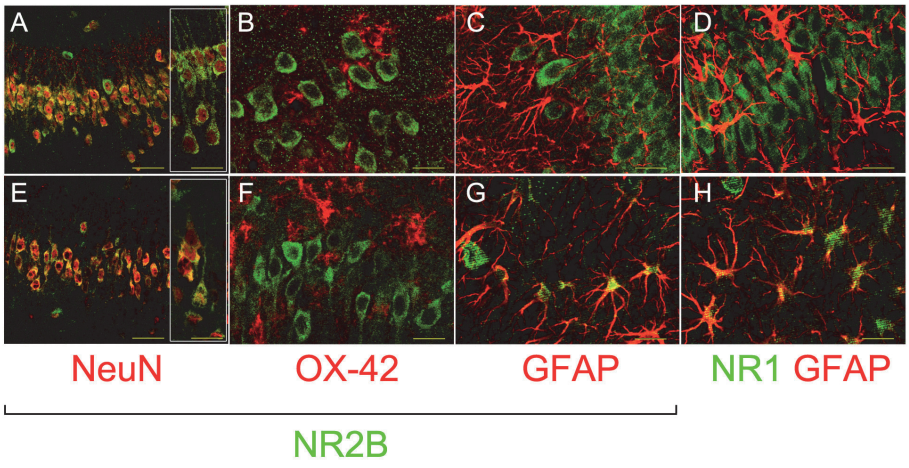


Figure 5.3 Ectopic localization of NR2B subunit in astrocytes during epileptogenesis.
Representative double-immunofluorescence micrographs showing the localization of NR2B in NeuN-positive neurons (A, E) in sham (A) and 96 h from SE onset (E). Panels B (sham) and F (epileptogenesis) depict lack of colocalization of NR2B with the microglia/macrophages marker OX-42; panels C and G show NR2B double-staining with GFAP, depicting receptor expression in astrocytes during epileptogenesis (G) but not in sham rats (C). Colocalization of NR2B and GFAP was found also in chronic epileptic rats (not shown). NR1 labeling (D,H) was observed in cells with neuronal morphology in sham rats (D), while it was additionally expressed by GFAP-positive astrocytes during epileptogenesis (H). Insets in A, E are high magnification of neurons expressing NR2B. Scale bar: A, E 50 µm; B-D, F-H and insets 20 µm.

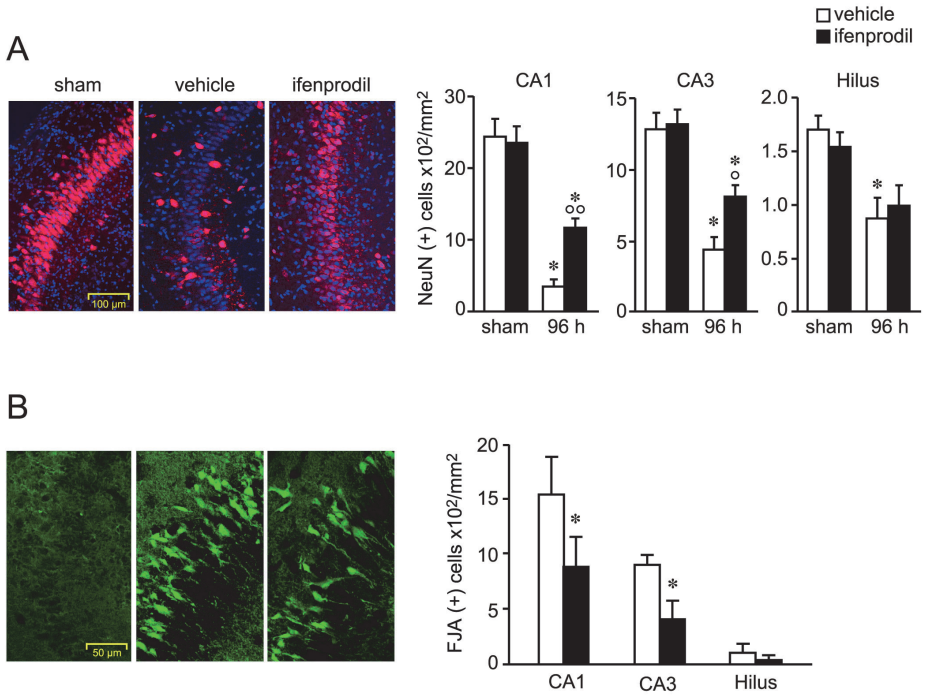


Figure 5.4 NR2B blockade during epileptogenesis mediates neuroprotection.
A. Representative confocal microscopy images of NeuN-Hoechst labeled CA3 sections from sham-implanted rats (sham), and from stimulated hippocampi of rats treated with vehicle or ifenprodil during epileptogenesis. Ifenprodil was injected 20 mg/kg, i.p. once daily for 3 days, starting 24 h after status epilepticus onset. Bargrams in A show quantification of neuronal density in the hippocampal subfields in the various experimental groups. *Sham*: rats implanted but not stimulated receiving vehicle (white, n=8) or ifenprodil (black, n=4); *96 h*: stimulated rats treated with vehicle (white, n=8) or ifenprodil (black, n=8) and killed during the epileptogenesis phase (i.e. 96 h from SE onset). Data are the mean ± SEM of number of neurons x 10^2 /mm^2 in CA1, CA3 and hilus. *p<0.01 vs sham groups; °p<0.05; °°p<0.01 vs. vehicle-96 h by two-way ANOVA followed by Tukey's test.
B. FJA-stained sections of CA3 region of the stimulated hippocampus from the same experimental groups described above. Bargrams report quantification of FJA positive cells in stimulated rats injected with vehicle (white) or treated with ifenprodil (black) and killed 96 h post-SE; sham groups are not included since no FJA positive cells were detected in control tissue. Data are the mean ± SEM of number of neurons x 10^2 /mm^2 in CA1, CA3 and hilus. *p<0.05 vs. vehicle-96 h by Student's t-test.

Role of redistributed NR2B-containing NMDARs during epileptogenesis

We addressed the pathophysiological role of pre-synaptic, extra-synaptic, and astrocytic NR2B-containing NMDARs during epileptogenesis by blocking these receptors with ifenprodil (Williams, 2001), then evaluating seizure-induced cell loss as read-out measure for excitotoxicity. Ifenprodil treatment resulted in significant

neuroprotection, as shown by the quantitative evaluation of NeuN-positive cell density in the stimulated hippocampus (Figure 5.4A). A significant decrease in neurons was induced by status epilepticus in CA1 (-85 ± 4%), CA3 (-65 ± 6%) and hilus (48 ± 11%), respectively 96 h from SE onset as compared to vehicle-injected sham rats ($p<0.01$). Ifenprodil significantly attenuated neuronal loss in CA1 (-52 ± 5%, $p<0.01$) and CA3 (-36 ± 6%, $p<0.05$). Conversely, no protective effect was observed in the hilus (-41% ± 10). Ifenprodil administration per se in sham rats did not change cell density in all regions analyzed (Figure 5.4A). The neuroprotective effect of ifenprodil was confirmed in the same brain specimens by evaluating degenerating FJA-positive cells (Figure 5.4B). Drug treatment reduced FJA-positive cells by 42 ± 17% and 54 ± 18% in CA1 and CA3, respectively ($p<0.05$), without significantly affecting hilar damage (Figure 5.4B).

Discussion

In this study, we provide new findings on the changes in the phosphorylation and localization of the NR2B subunit of the NMDAR in the rat hippocampus during epileptogenesis.

Using a rat model of electrically induced status epilepticus evolving to spontaneous seizures, we obtained the following findings during the epileptogenesis phase: 1. There is a significant reduction in the levels of P-NR2B in the post-synaptic compartment; 2. The total levels of NR2B, NR1, and PSD-95 are concomitantly decreased in the post-synaptic membranes; 3. The NR2B subunit redistributes in neuronal membranes with an increased localization in extra-synaptic and pre-synaptic compartments, and a concomitant decrease at post-synaptic sites. Moreover, we observed NR2B ectopic expression in activated astrocytes; 4. Pharmacological blockade of NR2B-containing NMDARs significantly reduces excitotoxicity.

By comparing the acute phase of status epilepticus, the epileptogenesis phase, and the chronic phase of spontaneous seizures in the same epilepsy model, we provide novel information on the dynamic changes of the NR2B subunit during the epileptic process, that reflect the epileptic activity. Thus, while P-NR2B levels are increased in the post-synaptic membranes during status epilepticus (Huo, et al., 2006, Moussa, et al., 2001, Niimura, et al., 2005), these levels are significantly reduced at the end of status epilepticus and during epileptogenesis. Since phosphorylation of NR2B facilitates its interaction with PSD-95 by anchoring the NMDAR to the post-synaptic membrane, the decrease in P-NR2B at the end of status epilepticus is an index of translocation of NR2B-containing NMDARs from synaptic to extra-synaptic sites (Collingridge, et al., 2004, Salter and Kalia, 2004, Viviani, et al., 2007). Accordingly, during status epilepticus we found a μ-calpain-mediated cleavage of the C-terminal domain of NR2B, which undergoes Tyr1472 phosphorylation and interacts with PSD-95 (Supplementary Figure

S5.2). This mechanism may facilitate the subsequent mobilization of NMDARs from synaptic to extra-synaptic compartments (Steigerwald, et al., 2000).

In-depth NMDAR analysis during the epileptogenesis phase supports increased receptor expression at extra-synaptic sites: 1. We found a drastic reduction in NR2B protein coupled to PSD-95, a synaptic NMDAR-associated scaffolding protein (El-Husseini, et al., 2000); 2. CREB phosphorylation, an intracellular signaling pathway predominantly activated by synaptic NMDARs (Hardingham and Bading, 2002) was reduced; 3. The proportion of [3H]-ifenprodil binding to NR2B-containing NMDAR in PSD was decreased; 4. Electron microscopy confirmed the reduction of NR2B in PSD, and showed increased NR2B localization both in extra-synaptic (Petralia, et al., 2010) and in pre-synaptic (Jourdain, et al., 2007) compartments.

The presence of extra-synaptic and pre-synaptic NR2B-containing NMDARs during epileptogenesis may affect the efficacy of synaptic transmission by at least two distinct mechanisms: 1. The extra-synaptic NMDARs may contribute to neuronal synchronization via activation of slow inward currents (Halassa, et al., 2007). These currents are increased in models of seizures and their pharmacological inhibition significantly attenuates the strength of ictal events (Bezzi and Volterra, 2001, Ding, et al., 2007, Fellin, et al., 2004); 2. The pre-synaptic NMDARs may increase glutamate release from neurons (Bezzi and Volterra, 2001, Jourdain, et al., 2007, Martin, et al., 1991) thus contributing to hyperexcitability by favoring the overactivation of post-synaptic glutamate receptors.

Notably, we provide new evidence that NR2B is expressed also by activated astrocytes during epileptogenesis, and the concomitant expression of the NR1 subunit indicates the presence of functional NMDARs in glia. NR2B expression in astrocytes was found also after transient forebrain ischemia, a brain injury also associated with excitotoxicity (Gottlieb and Matute, 1997, Krebs, et al., 2003). Although the function of NR2B-containing NMDARs in astrocytes needs further investigation, there is evidence of their involvement in the regulation of gliotransmission, thus suggesting they may indirectly affect neuronal excitability and viability by increasing the release of glutamate and D-serine (Haydon, 2001).

Short- and long-term NR2B subunit changes after spontaneous seizure occurrence in epileptic rats mirror the changes observed during and after status epilepticus, namely the subunit phosphorylation increases shortly after spontaneous seizures while it is reduced below control levels 24 h later. Although we did not study receptor localization by electron microscopy in these epileptic rats, the reduction in phosphorylated NR2B long term after seizures suggest that this subunit is increased extrasynaptically (and/or presynaptically) in the interictal phase. Conversely, the upregulation of NR2B phosphorylation during and shortly after seizures indicate the presence of PDS-anchored post-synaptic receptors, therefore denoting dynamic changes in the synaptic vs. extra-synaptic NMDAR during the ictal and interictal phases. The crucial role of the NR2B subunit in controlling neuronal excitability in chronic epileptic tissue is demonstrated by our recent evidence showing strong

reduction in recurrent spontaneous epileptic activity in mice treated with ifenprodil (Maroso et al., 2010). Accordingly, NR2B-selective antagonism during in vitro synchronous network activity in the CA3 area, reduces the probability of further epileptiform activity, likely due to depotentiation of the active synapses (Hellier, et al., 2009).

The reduction in NR2B, NR1, and PSD-95 in the post-synaptic membranes throughout the epileptic process likely reflects dendritic spine loss (El-Husseini, et al., 2000, Zha, et al., 2005) and progressive neurodegeneration induced by status epilepticus. We cannot exclude, however, that extra-synaptic translocation of NMDAR can, at least in part, contribute to the reductions in NR1 and NR2B levels. Indeed, we demonstrated a loss of NR2B expressing neurons by immunohistochemistry during the epileptogenesis phase. Therefore, our data indicate that pyramidal cells surviving to damage, and granule cells, which are spared from excitotoxicity, express NR2B predominantly at extra-synaptic sites, and these misplaced receptors may contribute to perpetuate hyperexcitability and excitotoxicity.

Pharmacological blockade of NR2B-containing NMDARs using ifenprodil during epileptogenesis significantly reduced neuronal cell loss in CA1 and CA3 pyramidal layers but not in the hilus in accordance with prominent expression of the NR2B subunit in pyramidal neurons (Monyer, et al., 1994). This evidence demonstrates that the changes occurring in NR2B subunit have functional consequences for excitotoxicity. It is possible that blockade of astrocytic NR2B-containing NMDARs may play a role in this neuroprotective effect (Krebs, et al., 2003). Although ifenprodil was previously shown to reduce cell loss in mice exposed to pilocarpine (Ding, et al., 2007), this pharmacological experiment was instrumental to establish a causal link between the changes in NR2B expression and localization during epileptogenesis and the neurodegenerative process.

Since NMDARs at extra-synaptic sites are involved in neuronal damage after experimental status epilepticus and stroke (Tu, et al., 2010), attempts to selectively antagonize these receptors, or strategies to prevent their extra-synaptic translocation or Ca2+ permeability function with specific cell permeable peptides (Tu et al, 2010) could be promising therapeutic approaches to attain neuroprotection. Thus, various brain injuries associated with the occurrence of symptomatic seizures and a higher risk of developing epilepsy (i.e. infection, neurotrauma, stroke, febrile seizures, status epilepticus) could be ideally targeted with this approach.

Cognitive dysfunctions might also be positively affected by blockade of NR2B containing NMDARs due to their neuroprotective effects (Rice, et al., 1998, Walker, 2007). In this frame, the antagonistic action of ifenprodil on extra-synaptic NMDARs would be advantageous since these receptors do not contribute to learning and memory functions (Bear and Malenka, 1994).

Finally, as regard the consequences of NMDAR blockade on epileptogenesis, one study showed no effect of a single intraventricular injection of ifenprodil given before status epilepticus on the percentage of rats developing epilepsy (Chen, et al., 2007); however,

our data indicate that such a treatment should be applied during epileptogenesis (i.e. at termination of status epilepticus) in order to specifically block the misplaced NR2B-containing NMDARs. Further attempts are therefore warranted to study if NR2B antagonism mediates antiepileptogenic effects in experimental models.

Conclusion

The present study provides novel information on the changes in the phosphorylation and localization of the NR2B subunit of the NMDAR in the rat hippocampus during the epileptogenesis phase prodromal to epilepsy development. Our data indicate that the NR2B subunit redistributes in neuronal membranes with an increased localization in extra-synaptic and pre-synaptic compartments, and a concomitant decrease at post-synaptic sites. Moreover, we observed NR2B ectopic expression in activated astrocytes. Pharmacological blockade of NR2B-containing NMDARs significantly reduces neuronal cell loss, indicating that the changes occurring in NR2B subunit have functional consequences for excitotoxicity, therefore could be targeted to attain neuroprotection after brain injuries.

References

Ali, D. W., and Salter, M. W., 2001. NMDA receptor regulation by Src kinase signalling in excitatory synaptic transmission and plasticity. Curr Opin Neurobiol 11, 336-342.

Araujo, I. M., Gil, J. M., Carreira, B. P., Mohapel, P., Petersen, A., Pinheiro, P. S., Soulet, D., Bahr, B. A., Brundin, P., and Carvalho, C. M., 2008. Calpain activation is involved in early caspase-independent neurodegeneration in the hippocampus following status epilepticus. J Neurochem 105, 666-676.

Auzmendi, J., Gonzalez, N., and Girardi, E., 2009. The NMDAR subunit NR2B expression is modified in hippocampus after repetitive seizures. Neurochem Res 34, 819-826.

Bear, M. F., and Malenka, R. C., 1994. Synaptic plasticity: LTP and LTD. Curr Opin Neurobiol 4, 389-399.

Bezzi, P., and Volterra, A., 2001. A neuron-glia signalling network in the active brain. Curr Opin Neurobiol 11, 387-394.

Bliss, T. V., and Collingridge, G. L., 1993. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361, 31-39.

Chatterton, J. E., Awobuluyi, M., Premkumar, L. S., Takahashi, H., Talantova, M., Shin, Y., Cui, J., Tu, S., Sevarino, K. A., Nakanishi, N., Tong, G., Lipton, S. A., and Zhang, D., 2002. Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. Nature 415, 793-798.

Chen, Q., He, S., Hu, X. L., Yu, J., Zhou, Y., Zheng, J., Zhang, S., Zhang, C., Duan, W. H., and Xiong, Z. Q., 2007. Differential roles of NR2A- and NR2B-containing NMDA receptors in activity-dependent brain-derived neurotrophic factor gene regulation and limbic epileptogenesis. J Neurosci 27, 542-552.

Choi, D. W., and Rothman, S. M., 1990. The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Annu Rev Neurosci 13, 171-182.

Collingridge, G. L., Isaac, J. T., and Wang, Y. T., 2004. Receptor trafficking and synaptic plasticity. Nat Rev Neurosci 5, 952-962.

Colonnier, M., 1968. Synaptic patterns on different cell types in the different laminae of the cat visual cortex. An electron microscope study. Brain Res 9, 268-287.

Conti, F., and Weinberg, R. J., 1999. Shaping excitation at glutamatergic synapses. Trends Neurosci 22, 451-458.

De Simoni, M. G., Perego, C., Ravizza, T., Moneta, D., Conti, M., Marchesi, F., De Luigi, A., Garattini, S., and Vezzani, A., 2000. Inflammatory cytokines and related genes are induced in the rat hippocampus by limbic status epilepticus. Eur J Neurosci 12, 2623-2633.

Ding, S., Fellin, T., Zhu, Y., Lee, S. Y., Auberson, Y. P., Meaney, D. F., Coulter, D. A., Carmignoto, G., and Haydon, P. G., 2007. Enhanced astrocytic Ca2+ signals contribute to neuronal excitotoxicity after status epilepticus. J Neurosci 27, 10674-10684.

Dingledine, R., Borges, K., Bowie, D., and Traynelis, S. F., 1999. The glutamate receptor ion channels. Pharmacol Rev 51, 7-61.

Dingledine, R., McBain, C. J., and McNamara, J. O., 1990. Excitatory amino acid receptors in epilepsy. Trends Pharmacol Sci 11, 334-338.

El-Husseini, A. E., Schnell, E., Chetkovich, D. M., Nicoll, R. A., and Bredt, D. S., 2000. PSD-95 involvement in maturation of excitatory synapses. Science 290, 1364-1368.

Fellin, T., Pascual, O., Gobbo, S., Pozzan, T., Haydon, P. G., and Carmignoto, G., 2004. Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. Neuron 43, 729-743.

Forder, J. P., and Tymianski, M., 2009. Postsynaptic mechanisms of excitotoxicity: Involvement of postsynaptic density proteins, radicals, and oxidant molecules. Neuroscience 158, 293-300.

Fumagalli, F., Frasca, A., Racagni, G., and Riva, M. A., 2008. Dynamic regulation of glutamatergic postsynaptic activity in rat prefrontal cortex by repeated administration of antipsychotic drugs. Mol Pharmacol 73**,** 1484-1490.

Gardoni, F., Mauceri, D., Malinverno, M., Polli, F., Costa, C., Tozzi, A., Siliquini, S., Picconi, B., Cattabeni, F., Calabresi, P., and Di Luca, M., 2009. Decreased NR2B subunit synaptic levels cause impaired long-term potentiation but not long-term depression. J Neurosci 29**,** 669-677.

Gardoni, F., Picconi, B., Ghiglieri, V., Polli, F., Bagetta, V., Bernardi, G., Cattabeni, F., Di Luca, M., and Calabresi, P., 2006. A critical interaction between NR2B and MAGUK in L-DOPA induced dyskinesia. J Neurosci 26**,** 2914-2922.

Gottlieb, M., and Matute, C., 1997. Expression of ionotropic glutamate receptor subunits in glial cells of the hippocampal CA1 area following transient forebrain ischemia. J Cereb Blood Flow Metab 17**,** 290-300.

Grimwood, S., Richards, P., Murray, F., Harrison, N., Wingrove, P. B., and Hutson, P. H., 2000. Characterisation of N-methyl-D-aspartate receptor-specific [(3)H]Ifenprodil binding to recombinant human NR1a/NR2B receptors compared with native receptors in rodent brain membranes. J Neurochem 75**,** 2455-2463.

Groc, L., Bard, L., and Choquet, D., 2009. Surface trafficking of N-methyl-D-aspartate receptors: physiological and pathological perspectives. Neuroscience 158**,** 4-18.

Halassa, M. M., Fellin, T., and Haydon, P. G., 2007. The tripartite synapse: roles for gliotransmission in health and disease. Trends Mol Med 13**,** 54-63.

Hardingham, G. E., and Bading, H., 2002. Coupling of extrasynaptic NMDA receptors to a CREB shut-off pathway is developmentally regulated. Biochim Biophys Acta 1600**,** 148-153.

Haydon, P. G., 2001. GLIA: listening and talking to the synapse. Nat Rev Neurosci 2**,** 185-193.

Hellier, J. L., White, A., Williams, P. A., Edward Dudek, F., and Staley, K. J., 2009. NMDA receptor-mediated long-term alterations in epileptiform activity in experimental chronic epilepsy. Neuropharmacology 56**,** 414-421.

Huo, J. Z., Dykstra, C. M., and Gurd, J. W., 2006. Increase in tyrosine phosphorylation of the NMDA receptor following the induction of status epilepticus. Neurosci Lett 401**,** 266-270.

Jourdain, P., Bergersen, L. H., Bhaukaurally, K., Bezzi, P., Santello, M., Domercq, M., Matute, C., Tonello, F., Gundersen, V., and Volterra, A., 2007. Glutamate exocytosis from astrocytes controls synaptic strength. Nat Neurosci 10**,** 331-339.

Kohl, B. K., and Dannhardt, G., 2001. The NMDA receptor complex: a promising target for novel antiepileptic strategies. Curr Med Chem 8**,** 1275-1289.

Krebs, C., Fernandes, H. B., Sheldon, C., Raymond, L. A., and Baimbridge, K. G., 2003. Functional NMDA receptor subtype 2B is expressed in astrocytes after ischemia in vivo and anoxia in vitro. J Neurosci 23**,** 3364-3372.

Langer, S. Z., 2008. Presynaptic autoreceptors regulating transmitter release. Neurochem Int 52**,** 26-30.

Lubisch, W., Beckenbach, E., Bopp, S., Hofmann, H. P., Kartal, A., Kastel, C., Lindner, T., Metz-Garrecht, M., Reeb, J., Regner, F., Vierling, M., and Moller, A., 2003. Benzoylalanine-derived ketoamides carrying vinylbenzyl amino residues: discovery of potent water-soluble calpain inhibitors with oral bioavailability. J Med Chem 46**,** 2404-2412.

Majores, M., Schoch, S., Lie, A., and Becker, A. J., 2007. Molecular neuropathology of temporal lobe epilepsy: complementary approaches in animal models and human disease tissue. Epilepsia 48 Suppl 2**,** 4-12.

Martin, D., Bustos, G. A., Bowe, M. A., Bray, S. D., and Nadler, J. V., 1991. Autoreceptor regulation of glutamate and aspartate release from slices of the hippocampal CA1 area. J Neurochem 56**,** 1647-1655.

Mody, I., and MacDonald, J. F., 1995. NMDA receptor-dependent excitotoxicity: the role of intracellular Ca2+ release. Trends Pharmacol Sci 16**,** 356-359.

Monyer, H., Burnashev, N., Laurie, D. J., Sakmann, B., and Seeburg, P. H., 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12**,** 529-540.

Moussa, R. C., Ikeda-Douglas, C. J., Thakur, V., Milgram, N. W., and Gurd, J. W., 2001. Seizure activity results in increased tyrosine phosphorylation of the N-methyl-D-aspartate receptor in the hippocampus. Brain Res Mol Brain Res 95**,** 36-47.

Niimura, M., Moussa, R., Bissoon, N., Ikeda-Douglas, C., Milgram, N. W., and Gurd, J. W., 2005. Changes in phosphorylation of the NMDA receptor in the rat hippocampus induced by status epilepticus. J Neurochem 92**,** 1377-1385.

Noe, F., Pool, A. H., Nissinen, J., Gobbi, M., Bland, R., Rizzi, M., Balducci, C., Ferraguti, F., Sperk, G., During, M. J., Pitkanen, A., and Vezzani, A., 2008. Neuropeptide Y gene therapy decreases chronic spontaneous seizures in a rat model of temporal lobe epilepsy. Brain 131**,** 1506-1515.

Papadia, S., and Hardingham, G. E., 2007. The dichotomy of NMDA receptor signaling. Neuroscientist 13**,** 572-579.

Paxinos, G., and Watson, C., 1986. The rat brain in stereotaxic coordinates. Academic Press, New York.

Petralia, R. S., Wang, Y. X., Hua, F., Yi, Z., Zhou, A., Ge, L., Stephenson, F. A., and Wenthold, R. J., 2010. Organization of NMDA receptors at extrasynaptic locations. Neuroscience 167**,** 68-87.

Pozzi, L., Hakansson, K., Usiello, A., Borgkvist, A., Lindskog, M., Greengard, P., and Fisone, G., 2003. Opposite regulation by typical and atypical anti-psychotics of ERK1/2, CREB and Elk-1 phosphorylation in mouse dorsal striatum. J Neurochem 86**,** 451-459.

Ravizza, T., Gagliardi, B., Noe, F., Boer, K., Aronica, E., and Vezzani, A., 2008. Innate and adaptive immunity during epileptogenesis and spontaneous seizures: evidence from experimental models and human temporal lobe epilepsy. Neurobiol Dis 29**,** 142-160.

Ravizza, T., and Vezzani, A., 2006. Status epilepticus induces time-dependent neuronal and astrocytic expression of interleukin-1 receptor type I in the rat limbic system. Neuroscience 137**,** 301-308.

Rice, A. C., and DeLorenzo, R. J., 1998. NMDA receptor activation during status epilepticus is required for the development of epilepsy. Brain Res 782**,** 240-247.

Rice, A. C., Floyd, C. L., Lyeth, B. G., Hamm, R. J., and DeLorenzo, R. J., 1998. Status epilepticus causes long-term NMDA receptor-dependent behavioral changes and cognitive deficits. Epilepsia 39**,** 1148-1157.

Salter, M. W., and Kalia, L. V., 2004. Src kinases: a hub for NMDA receptor regulation. Nat Rev Neurosci 5**,** 317-328.

Schmitz, C., and Hof, P. R., 2005. Design-based stereology in neuroscience. Neuroscience 130**,** 813-831.

Sierra-Paredes, G., and Sierra-Marcuno, G., 2007. Extrasynaptic GABA and glutamate receptors in epilepsy. CNS Neurol Disord Drug Targets 6**,** 288-300.

Steigerwald, F., Schulz, T. W., Schenker, L. T., Kennedy, M. B., Seeburg, P. H., and Kohr, G., 2000. C-Terminal truncation of NR2A subunits impairs synaptic but not extrasynaptic localization of NMDA receptors. J Neurosci 20**,** 4573-4581.

Sun, Q. J., Duan, R. S., Wang, A. H., Shang, W., Zhang, T., Zhang, X. Q., and Chi, Z. F., 2009. Alterations of NR2B and PSD-95 expression in hippocampus of kainic acid-exposed rats with behavioural deficits. Behav Brain Res 201**,** 292-299.

Tovar, K. R., and Westbrook, G. L., 2002. Mobile NMDA receptors at hippocampal synapses. Neuron 34**,** 255-264.

Tu, W., Xu, X., Peng, L., Zhong, X., Zhang, W., Soundarapandian, M. M., Balel, C., Wang, M., Jia, N., Lew, F., Chan, S. L., Chen, Y., and Lu, Y., 2010. DAPK1 interaction with NMDA receptor NR2B subunits mediates brain damage in stroke. Cell 140**,** 222-234.

Viviani, B., Gardoni, F., and Marinovich, M., 2007. Cytokines and neuronal ion channels in health and disease. Int Rev Neurobiol 82**,** 247-263.

Walker, M., 2007. Neuroprotection in epilepsy. Epilepsia 48 Suppl 8**,** 66-68.

Williams, K., 2001. Ifenprodil, a novel NMDA receptor antagonist: site and mechanism of action. Curr Drug Targets 2**,** 285-298.

Woodhall, G., Evans, D. I., Cunningham, M. O., and Jones, R. S., 2001. NR2B-containing NMDA autoreceptors at synapses on entorhinal cortical neurons. J Neurophysiol 86**,** 1644-1651.

Wyneken, U., Marengo, J. J., Villanueva, S., Soto, D., Sandoval, R., Gundelfinger, E. D., and Orrego, F., 2003. Epilepsy-induced changes in signaling systems of human and rat postsynaptic densities. Epilepsia 44**,** 243-246.

Yang, J., Woodhall, G. L., and Jones, R. S., 2006. Tonic facilitation of glutamate release by presynaptic NR2B-containing NMDA receptors is increased in the entorhinal cortex of chronically epileptic rats. J Neurosci 26**,** 406-410.

Zha, X. M., Green, S. H., and Dailey, M. E., 2005. Regulation of hippocampal synapse remodeling by epileptiform activity. Mol Cell Neurosci 29**,** 494-506.

Table S5.1 Study design and number of rats used in each experiment.

Experimental group	WB	IHC	IP	Binding	EM	In vivo pharmcacology	
Sham	14	8	3	18	2	(A-705253)	(Ifenprodil)
						6	12
SE - 2h	4	4					
SE -18h	5	4				12	
SE - 96h	6	5	3	18	2		16
SRS -2h	6	4					
SRS -24h	5	5					

The total number of rats used in the different experiments is shown. *A-705253*: 6 rats in each experimental group. *Ifenprodil*: 8 sham and 8 stimulated rats treated with vehicle; 4 sham and 8 stimulated rats treated with ifenprodil. WB=western blot; IHC=immunohistochemistry; IP=immunoprecipitation; EM=immuno-electron microscopy; SE=status epilepticus; SRS=spontaneous recurrent seizures.

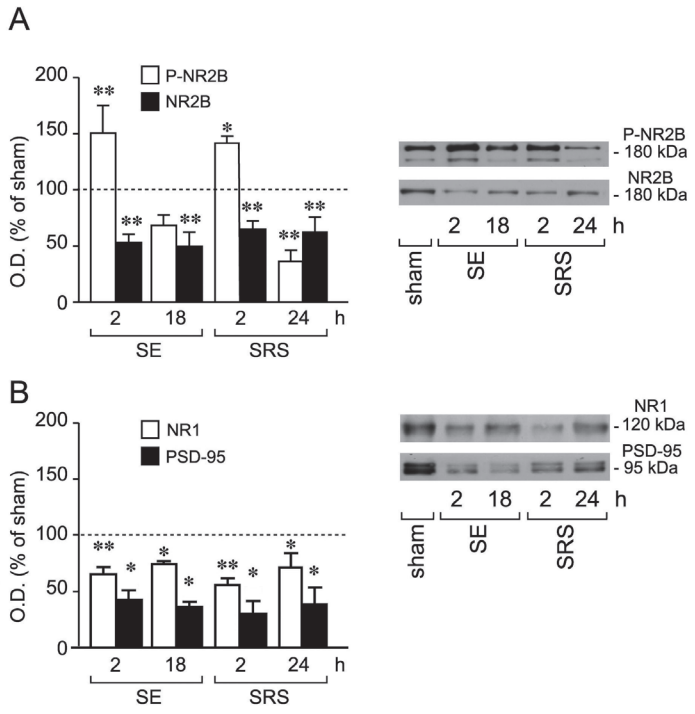


Figure S5.1 Differential changes in NR2B, NR1 and PSD-95 during acute and chronic seizures.
Western blot analysis of Tyr^{1472} phosphorylated NR2B and NR2B protein levels (A) and NR1 and PSD-95 (B) in the PSD-enriched fraction of the stimulated hippocampus at different time points after the onset of electrical status epilepticus (2 and 18 h SE) and after a spontaneous seizure (2 and 24 h SRS).
Bargrams show mean ± SEM (n=4-6 each experimental rats; n=14 sham rats) of the optical density (O.D) values of relevant bands divided by the corresponding β-actin (internal standard). Data are expressed as percentage of control values (sham rats). Representative bands of each protein are depicted in panels A,B with their corresponding molecular weight. *p<0.05; **p<0.01 by one-way ANOVA followed by Dunnett's test (statistical analysis includes values at 96 h depicted in Fig. 1 of main text).

μ-Calpain-induced NR2B breakdown

To give insights into the mechanisms underlying the translocation of NR2B to extra-synaptic sites during epileptogenesis, we assessed whether this subunit was cleaved at its C-terminal domain by the activated protease μ-calpain. This domain contains the Tyr^{1472} phosphorylation site of the NR2B subunit, which interacts with the synaptic scaffolding protein PSD-95 (Steigerwald, et al., 2000).

Western blot

Stimulated hippocampi (n=4 exp rats; n=14 sham rats; Supplementary Table S5.1) were homogenized and used for the subcellular fractionation (Gardoni, et al., 2009).
The post-synaptic density (PSD)-enriched fraction was used to measure both the full-length NR2B (180 kDa) (1:1000) and the cleaved protein fragment (115kDa) (1:500) using an anti-NR2B rabbit polyclonal antibody (Zymed Laboratories).
The cytosolic component obtained from the first 2 sequential centrifugations was used to detect μ-calpain. Fifty μg protein in each sample were separated by gel electrophoresis (8% SDS-PAGE) under reducing conditions, and each sample was run in duplicate. Proteins were transferred to Hybond nitrocellulose membranes (Bio-Rad Laboratories) by electroblotting, then active (76 kDa) and inactive (78 kDa) μ-calpain were measured by using anti-μ-calpain I mouse monoclonal antibody (1:500; Chemicon).

A-705253 treatment

A different group of rats was treated with the selective μ-calpain inhibitor A-705253 (a gift from Abbott, Ludwigshafen, Germany) (Lubisch, et al., 2003). The drug was dissolved in saline (pH ~5.5) and injected 10 mg/kg, intraperitoneally (i.p.) 1 h before starting the electrical stimulation, then 8 h and 17 h after SSLSE onset (n=6, Supplementary Table S5.1). Rats were killed for western blot analysis 1 h after the last injection. Control rats were sham-implanted and injected with vehicle (n=6). Treatment with A-705253 did not interfere with behavioral seizures during the electrical stimulation or with status epilepticus duration. Thus, both control and the experimental groups experienced generalized seizures within the 4^{th} epoch of stimulation; the duration of SSLSE as assessed by EEG analysis was not statistically different in the two experimental groups (not shown). End of status epilepticus was considered when the synchronized pattern of spiking activity elapsed (i.e. ≤18 h).

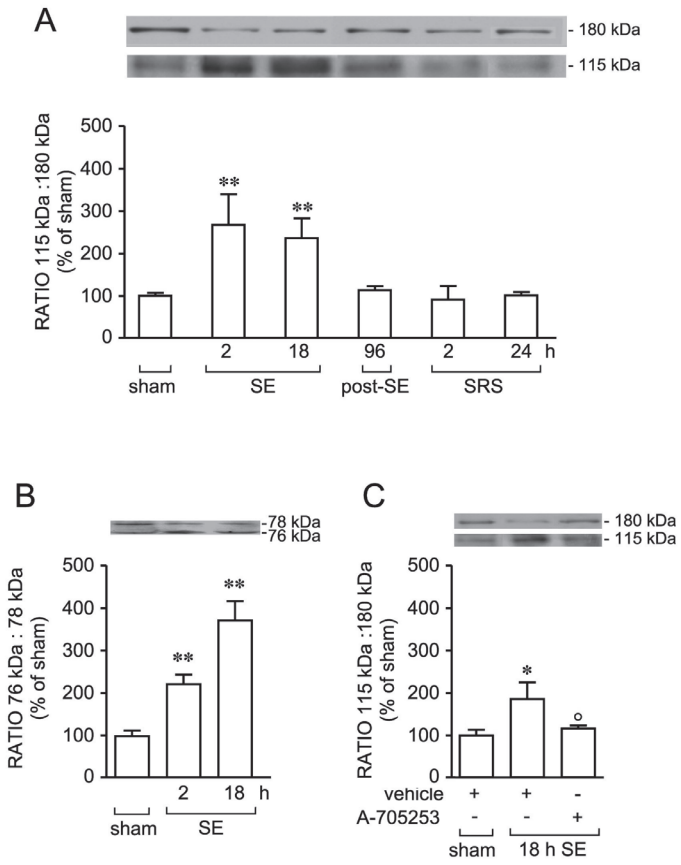


Figure S5.2 Western blot analysis of NR2B breakdown and μ-calpain activation during status epilepticus.
A. NR2B breakdown was measured by the ratio between the cleaved 115 kDa and the full length 180 kDa form of this receptor subunit in the stimulated hippocampus at different time points after the onset of electrical status epilepticus. NR2B breakdown was significantly increased during the acute phase of SE (2 h: +169 ± 71%; 18 h: +135 ± 50%, $p<0.01$), while it did not change in the subsequent phases of the epileptic process.
B. μ-calpain activation, measured by the ratio between the active 76 kDa and the inactive 78 kDa forms of this protein, significantly increased both 2 h (+123 ± 20%, $p<0.01$) and 18 h (+309 ± 44%, $p<0.01$) after status epilepticus.
C. The effect of μ-calpain inhibition on NR2B breakdown during status epilepticus was measured by injecting rats with A-705253 (10 mg/kg, i.p., n=6) beginning the treatment 60 min before electrical stimulation, then injecting the inhibitor 8 h and 17 h thereafter. Rats were killed 60 min after the last drug administration. Vehicle-18 h SE group represents rats stimulated and injected with saline using the same protocol adopted for A-705253 (n=6). Sham rats were rats injected with vehicle but not stimulated (n=6). Treatment of rats with A-705253, a specific μ-calpain inhibitor, prevented the cleavage of NR2B subunit.
Bargrams show mean ± SEM of the optical density (O.D) values. The O.D. of the relevant bands was divided by the corresponding β-actin (internal standard). Data are expressed as percentage of control values (sham rats). Representative bands of each protein are depicted in panels A-C with their corresponding molecular weight.
*$p<0.05$; **$p<0.01$ vs sham groups; °$p<0.05$ vs. vehicle-18 h SE by one-way ANOVA followed by Dunnett's (panels A, B) or Tukey's test (panel C).

Chapter 6

Vagus nerve stimulation in children with intractable epilepsy: a randomized controlled trial

S. Klinkenberg*, M. Aalbers*, J. Vles, E. Cornips, K. Rijkers, L. Leenen, A. Kessels, A. Aldenkamp, M. Majoie
* These authors contributed equally

Developmental Medicine & Child neurology 2012;54:855-61

Abstract

We aimed to evaluate the effects of vagus nerve stimulation (VNS) in children with intractable epilepsy on seizure frequency and severity and in terms of tolerability and safety.

In this study, the first randomized active controlled trial of its kind in children, 41 children (23 boys; mean age at implantation 11 years 2 months, standard deviation 4 years 2 months) were included. Thirty-five patients suffered from localization-related epilepsy (25 symptomatic, 10 cryptogenic), while 6 patients had generalized epilepsy (4 symptomatic, 2 idiopathic).

During a baseline period of 12 weeks, seizure frequency and severity were recorded using seizure diaries and the adapted Chalfont Seizure Severity Scale (NHS3), after which the patients entered a blinded active controlled phase of 20 weeks. During this phase, half of the patients received high output VNS (maximally 1.75 mA) and the other half received low output stimulation (0.25 mA). Finally, all patients received high output stimulation for 19 weeks. For both phases, seizure frequency and severity were assessed as during the baseline phase. Overall satisfaction, and adverse events were assessed by semi-structured interviews.

At the end of the randomized controlled blinded phase, seizure frequency reduction of 50% or more occurred in 16% in the high output stimulation group and in 21% in the low output stimulation group ($p=1.00$). There was no significant difference in decrease of seizure severity between patients in both stimulation groups. Overall, VNS reduced seizure frequency by 50% or more in 26% of patients at the end of the add-on phase. The overall seizure severity also improved ($p=<0.001$).

VNS is a safe and well-tolerated adjunctive treatment of epilepsy in children. Our results suggest that the effect of VNS on seizure frequency in children is limited. However, the possible reduction in seizure severity and improvement in well-being makes this treatment worth considering in the individual child with intractable epilepsy.

Introduction

Vagus nerve stimulation (VNS) is a neuromodulatory treatment that is used as an adjunctive therapy for individuals with medically refractory epilepsy who are not eligible for epilepsy surgery or in whom surgery has failed, and in whom non-epileptic events are excluded. VNS consists of chronic intermittent electrical stimulation of the vagus nerve, delivered by a programmable pulse generator.

Randomized active-controlled trials, which have predominantly included adults, have demonstrated the safety and efficacy of VNS (Handforth, et al., 1998, The VNS Study Group, 1995): seizure frequency decreased by 50% or more in 23-31% of patients in the treatment group compared to 13-15% in the placebo group. These trials led to the U.S. Food and Drug Administration approval in 1997 of the use of VNS as adjunctive therapy in individuals older than 12 years with partial epilepsy refractory to treatment with available antiepileptic drugs (AEDs).

The effectiveness of VNS might be more variable in children than in adults. Numerous prospective and retrospective studies at various centres worldwide describing more than 650 children, aged 0-19 years, have reported a reduction in seizure frequency of 0-90% (Alexopoulos, et al., 2006, Arthur, et al., 2007, Benifla, et al., 2006, Blount, et al., 2006, Danielsson, et al., 2008, Helmers, et al., 2001, Hornig, et al., 1997, Kabir, et al., 2009, Kang, et al., 2006, Khurana, et al., 2007, Kossoff and Pyzik, 2004, Lundgren, et al., 1998, Majoie, et al., 2001, Majoie, et al., 2005, Murphy, 1999, Murphy, et al., 1995, Murphy, et al., 2000, Nagarajan, et al., 2002, Parain, et al., 2001, Parker, et al., 1999, Patwardhan, et al., 2000, Rossignol, et al., 2009, Rychlicki, et al., 2006, Saneto, et al., 2006, Shahwan, et al., 2009, Zamponi, et al.). However, these studies were uncontrolled, and there was a large variation in study groups for example regarding age, epilepsy syndromes, and follow-up duration, which varied from 3 months to 10 years.

No randomized active-controlled paediatric trial that unequivocally demonstrates the efficacy of VNS in children has yet been conducted. This study was carried out with the aim of evaluating the tolerability and effectiveness of VNS in children with intractable epilepsy in a randomized active-controlled study. Moreover, we sought to identify responder characteristics that may improve future patient selection.

Methods

Study design

This study was a randomized active controlled double-blinded add-on study. The study was divided into a baseline (12 weeks) and a blinded treatment phase (20 weeks). During the treatment phase patients received either high (therapeutic) or low (active control) stimulation. This active control group was incorporated to protect the

blinding, because patients can detect stimulation. Additionally, after the blinded phase all patients participated in a non-controlled follow up, in which they received high stimulation (19 weeks; add-on phase).
All parents, guardians, and patients aged 12 years or above gave informed consent, and all procedures were approved by the ethics committee of Maastricht University Medical Centre.

Patients

41 Children with medically refractory epilepsy participated in the study. Inclusion criteria were:
- Medically refractory epilepsy despite adequate and stable AED concentrations
- Age between 4 and 18 years at implantation
- Not eligible for epilepsy surgery
- Written and signed informed consent from parents or guardians

Exclusion criteria included the following:
- Non-epileptic seizures
- Documented history of generalized status epilepticus in the previous three months
- Evidence of a progressive cerebral lesion, degenerative disorder, malignancy in the previous 5 years
- Unstable medical disease (i.e. cardiovascular, hepatic, renal, musculoskeletal, gastrointestinal, metabolic, endocrine) in the previous 2 years
- Schizophrenia or any psychotic symptomatology
- High risks of complications (obstructive respiratory disease, gastric disorders, cardiac rhythm disorders)
- History of alcohol or drug abuse, psychiatric disorder requiring electro-convulsive therapy, or chronic use of major tranquillizers (neuroleptics, antidepressants, or MAO inhibitors) in the previous 6 months
- Regular treatment with antihistamines, metoclopramide, or central nervous system-active compounds
- Treatment with an experimental drug during the previous 30 days

Device and implantation

All surgical procedures were performed at the Maastricht University Medical Centre. Bipolar electrodes were placed around the left vagus nerve and connected to the programmable pulse generator (Neurocybernetic prothesis NCP, Cyberonics Inc., Webster, TX, USA), which was implanted subcutaneously or underneath the pectoral muscle below the clavicle.

Randomization, blinding, and device settings

The treating neurologist (MM), patients, parents, and guardians were blinded to treatment conditions. Patients were allocated to a treatment condition by one trial nurse (LL) using a computer program. The trial nurse also adjusted device settings according to a fixed protocol: 2 weeks after surgery the device was set to the parameters depicted in Table 6.1. Thereafter, in the treatment group the current was increased stepwise at 2-week intervals to the maximally tolerated output current (maximum 1.75 mA) on the basis of the clinical evaluation of the treating neurologist. The stepwise increase was stopped when a patient experienced a seizure reduction of 50% or more or was delayed or set back to the highest tolerable level in case of side effects.
In the active control group, the output current was increased temporarily during each visit and switched back to the initial output current at the end of the visit. At the end of the blinded phase, the output parameters of the active control group were adjusted according to the schedule and parameters of the treatment group. The maximally applied current at the end of this phase was set at 2.25 mA for both groups.

Table 6.1 Initial device settings.

	Treatment group	Active control group
Output current (mA)	0.25	0.25
Pulse width (ms)	0.5	0.1
Frequency (Hz)	30	1
Duty cycle: on (sec)/ off (min)	30/5	14/60

Outcome parameters

Seizure frequency was recorded by patients' parents or guardians using a diary. Several seizure types were scored separately. Seizures were classified according to the ILAE classification (Commission on Classification and Terminology of the International League Against Epilepsy, 1989). Seizure frequency was recorded in the 12 weeks prior to implantation to serve as a baseline.
Seizure severity was estimated by administering the adapted Chalfont Severity Scales (NHS3) at baseline and at the end of the blinded and add-on phase to the caregiver who witnessed the seizures (O'Donoghue, et al., 1996). This scale includes seven seizure-related factors and generates a score from 1 to 27: the higher the score, the more severe the seizures.
Overall satisfaction was evaluated at the end of the non-blinded add-on phase by standardized questioning the parents or guardians.
Adverse events were recorded at each visit (12 in total) by questioning parents or guardians using a semi-structured interview.
IQ was assessed using the Peabody Picture Vocabulary test (PPVT-III-NL) (Dunn and Dunn, 2005) and the Beery VMI 5th edition (Beery and Beery, 2006), a developmental

test of visual motor integration. To calculate IQ, raw scores were converted to age-equivalents in months, which were divided by the age in months at the date of testing and multiplied by 100. Finally, the average of both tests was calculated.

Statistical analysis

Power analysis was performed based on the assumption that 40% and 5% of patients in treatment and control groups respectively, had a clinically relevant response, which was defined as 50% or more seizure frequency reduction. Assuming a level of significance of 0.05 (two-tailed) and a power of 80% the size of our study population was calculated at two times 18.
Statistical analysis was performed using SPSSv19 for MacOX.

Seizure frequency

Seizure frequency during the blinded phase was expressed as a percentage change compared to seizure frequency during baseline for each patient. Seizure frequency changes were classified into different categories (≥50% increase, no response (<50% increase or decrease), ≥50% decrease). The number of patients experiencing a 50% or more reduction in seizure frequency was compared between the high and low output group using Fisher's exact test. Furthermore, we compared the percentage change in seizure frequency between the low and high output group. First the percentage change was transformed by calculating the natural logarithm of each value, as these data were not normally distributed. The resulting values were normally distributed in both groups and therefore, were compared using the Student's t-test. The same analysis was repeated for the final 30 days of the blinded phase in order to assess any lag in efficacy or increased effectiveness over time. Overall improvement of seizure frequency was calculated by comparing the seizure frequency at the end of the add-on phase with baseline by a related-samples Wilcoxon signed rank test. Finally, seizure frequency changes were classified into different categories (≥50% increase, no response, ≥50% decrease).

Seizure severity

To asses seizure severity, first a mean NHS3 score was calculated for each patient by summing up the scores from each time point and dividing them by the number of seizure types. The median differences between groups were compared using a Mann-Whitney U test. Overall improvement of seizure severity was calculated by comparing the NHS3 score at the end of the add-on phase with baseline using a paired t-test.

Responder characteristics

We analyzed the relationship between the final outcome at the end of the add-on phase and the following parameters by correlation analysis (Kendall's tau): age at

implantation, age at onset, and number of AEDs that had been used before implantation. The Mann-Whitney U test was used for the variables sex and presence of bilateral interictal discharges, and the Kruskal-Wallis test was used for aetiology.
A p-value <0.05 was considered statistically significant.

Results

Demographics

Forty-one children were implanted with VNS after appropriate regulatory approval. Inclusion took place over a two-year period. The mean age at implantation was 11 years 2 months (range 3 years 10 months to 17 years 8 months, median 12 years 4 months) and twenty-three were boys. Seizure onset occurred at a mean age of 2 years 4 months (range 0-12 years, median 1 year 2 months)
Two patients had undergone unsuccessful epilepsy surgery several years before VNS. Patients had been exposed to a mean of 7.2 AEDs and were receiving a mean of 2.4 AEDs at implantation. Fifteen of the 41 children had been on the ketogenic diet.
The majority of patients had localization related epilepsy, which in 10 patients was cryptogenic and in 25 was symptomatic. Four patients had symptomatic generalized epilepsy and only two patients had idiopathic generalized epilepsy. All but three children had learning disabilities. Twenty-five patients had multiple seizure types and 16 patients had a single seizure type.
An overview of baseline characteristics of both groups is provided in Table 6.2. There were no significant differences at baseline between the high and low stimulation groups with regard to sex, age at implantation, age at onset, seizure frequency, ILAE classification (localization related: symptomatic, cryptogenic; generalized: idiopathic or symptomatic), or number of AEDs used before surgery.
Three out of 41 patients who entered the study were excluded from seizure frequency calculations because of unreliable/ incomplete seizure diaries. Two of the excluded patients were in the high and one was in the low stimulation group. Additionally, information for four patients regarding the last 30 days of the add-on phase was missing.

Table 6.2 Baseline characteristics of study groups.

	High	Low
Number of patients (male/ female)	21 (11/10)	20 (12/8)
Mean age at implantation (range)	10y11m (3y10m-17y8m)	11y6m (4y2m-17y2m)
Mean age at onset (range)	2y10m (0-12)	1y8m (0-5)
Median age at onset (range)	1y2m (0-12)	1y2m (0-5)
Mean interval onset-implantation (range)	7y8m (2-16)	9y5m (3-15)
Median seizure frequency at baseline (range), seizures/day	2.1 (0.1-53.7)	0.9 (0.1-31.7)
ILAE classification		
Localization related	19 (90%)	16 (80%)
Symptomatic	15 (71%)	10 (50%)
Cryptogenic	4 (19%)	6 (30%)
Generalized	2 (10%)	4 (20%)
Idiopathic	0	2 (10%)
Symptomatic	2 (10%)	2 (10%)
Mean number of AEDs	7.0 (5-10)	7.3 (4-14)

AED=antiepileptic drug; ILAE= International League Against Epilepsy; m=months; y=years.

Effects of low versus high stimulation – end of randomized controlled blinded phase

Seizure frequency

Three patients in the high group (16%) and four in the low group (21%) had a greater than 50% seizure frequency reduction (p=1.00; Figure 6.1A). Among patients receiving high stimulation, the median seizure frequency increased by 23.4% compared with baseline, whereas among patients receiving low stimulation, seizure frequency fell by a median of 8.8% (comparison log transformed values, p=0.63). Focussing on the last thirty days of the blinded phase, the median seizure frequency decrease was 3.1% and 5.1% in the high and low group respectively (comparison log transformed values, p=0.47).

Seizure severity

Patients in the high stimulation group had a mean decrease in NHS3 score of 0.3, while patients in the low stimulation group had a mean decrease of 0.6 (p=0.71).

Overall effects of stimulation – end of add-on phase

Seizure frequency

Nine out of 34 patients (26%) experienced a 50% or more seizure frequency reduction, five (15%) experienced a 50% or more increase, and 20 (59%) did not respond at all (Figure 6.1B). Seizure frequency decreased from a median of 1.61 seizures per day during the baseline phase to a median of 1.12 at the end of the add-on phase (p=0.02).

Seizure severity

Seizure severity improved from a mean score of 9.5 at baseline to a mean score of 8.3 at the end of the add-on phase (p=0.00) (Figure 6.1C).

Overall satisfaction

Seventy-eight percent of the patients (n=32) perceived some kind of positive effect at the end of the add-on phase.

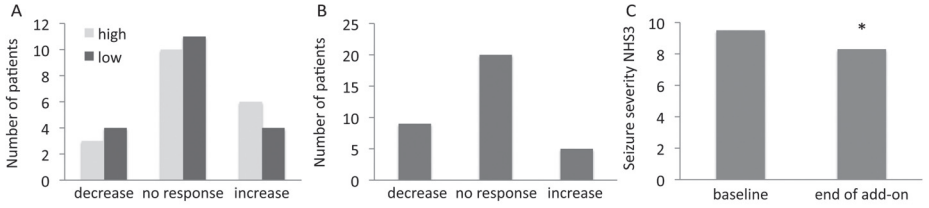


Figure 6.1 Effect of VNS on seizure frequency and severity.
A Response distribution for high and low stimulation group during double blind phase ≥50% increase, no response, ≥50% decrease; B Distribution overall response at the end of the add-on phase ≥50% decrease, no response, ≥50% increase; C Mean seizure severity score according to the Chalfont Seizure Severity Scale, baseline compared to end of add-on phase, * p<0.05.

Responder characteristics

There was weak evidence of a correlation between seizure frequency reduction at the end of the add-on phase and age at onset (τ= .21, p=0.08): children with a lower age at onset tended to respond better. Age at implantation and the number of AEDs that had been used before implantation were not correlated with seizure frequency reduction. No statistically significant differences in seizure frequency reduction were observed between boys and girls, between different aetiologies, and between the children with or without bilateral interictal activity.

Adverse events

The most frequently reported adverse events were voice alterations, coughing, and throat pain (Table 6.3). The majority of side effects were transient and most of them were stimulus related - some did not appear to be directly related to VNS. Reported behavioural changes consisted of agitation, crying, or frequent startles. Wound infection occurred in two patients. There was no need for device removal - both infections were successfully treated with short-term antibiotics. There were no other surgery-related side effects. Discontinuation during the study because of side effects did not occur.

Table 6.3 Adverse effects.

Adverse event	Number of patients
Voice alterations	8 (20%)
Coughing	3 (7%)
Throat pain	3 (7%)
Tingling sensations in throat	2 (5%)
Behavioural changes	3 (7%)
Infection	2 (5%)
Headache	1 (2%)
Spontaneous swelling around stimulator	1 (2%)
Pain around stimulator during exercise	1 (2%)
Itch	1 (2%)

Discussion

This study is the first randomized active controlled study on the effectiveness of VNS in children. We observed no statistically significant difference in seizure frequency reduction and seizure severity when comparing high and low stimulation groups. However, both seizure frequency and seizure severity at the end of the add-on phase were significantly decreased compared with baseline. A more than 50% seizure frequency reduction was reached in nine out of 34 patients (26,4%) at the end of the add-on phase. There was a trend towards a significant correlation between onset age and a favourable response to VNS. Strikingly, completion rate and overall satisfaction were very high, as stimulation was discontinued in only one out of 41 patients, and more than three-quarters of the parents and guardians reported some kind of improvement.
Our results confirm previous reports demonstrating that VNS in children is safe, when performed by experienced neurosurgeons: no major adverse events occurred and side effects of VNS were mild, mostly transient, and related to stimulation. Infection occurred in only two patients and was completely resolved with short-term antibiotics.

We were not able to demonstrate a favourable effect of high stimulation versus low stimulation as has been observed in adult randomized trials (Handforth, et al., 1998, The VNS Study Group, 1995). Several factors may account for this. First of all, the size of our population might have been too small. Owing to the lack of other randomized studies in children, the power analysis was based on open-label studies, which suggested a larger effect than in adults. According to the current results, future studies should include a larger study population. Second, there are differences in vagus nerve electrophysiology between adults and children: threshold currents are higher and conduction velocities are lower in younger children than in older children and adults, indicating that maturation of the vagus nerve is not yet completed in young children (Koo, et al., 2001). Moreover, the developing brain may respond differently to VNS. De

Herdt et al. observed lower efficacy of VNS in children as compared to adults as well (De Herdt, et al., 2007).
Our results particularly contrast with the findings of Murphy et al, who reported a median seizure frequency reduction of 23% after 3 months of stimulation. This difference may be explained by differences in experimental design: Murphy et al. included 41 patients from the Compassionate Protocol as well. As this protocol is uncontrolled, VNS effectiveness may have been overestimated. Indeed, several other studies observed no seizure frequency reduction in part of their study population (Kabir, et al., 2009, Khurana, et al., 2007, Majoie, et al., 2001, Majoie, et al., 2005, Parker, et al., 1999, Patwardhan, et al., 2000) or in the entire study population. (Arthur, et al., 2007, Danielsson, et al., 2008)

In our study, seizure frequency reduction in the lower output group was higher than expected. A decrease in seizure frequency may have resulted from the natural fluctuation of the disease which is probably higher in children than in adults (Parker, et al., 1999addendum). Indeed, children were twice as likely as adults to respond with a greater than 50% seizure frequency reduction during placebo treatment (Rheims, et al., 2008). Although active controlled treatment is not equivalent to placebo treatment, is it unlikely that low stimulation may yield a true effect, as previous studies have demonstrated that the chosen combination of current intensity and pulse width does not evoke an action potential (Heck, et al., 2002, Koo, et al., 2001).

Overall, VNS reduced seizure frequency by 50% or more in 26.4% patients. Effectiveness of VNS has possibly been influenced by several factors. First of all, it has been suggested that learning disability may be a negative predictor (Aldenkamp, et al., 2002, Danielsson, et al., 2008, Hallbook, et al., 2005, Rychlicki, et al., 2006). As all but three of our patients had learning disabilities, this may have contributed to the modest effect of VNS in our study. The chosen stimulation parameters may also have affected VNS efficacy. In our study, the output current was no higher than 1.75 mA during the blinded phase and 2.25 mA during the add-on phase in order to prevent demyelinisation of the nerve, which can occur even at normal output currents (1.5 mA, 250µs, 20 Hz) (Tubbs, et al., 2001). It is unlikely that a further increase of output current would have resulted in a larger effect of VNS, as our results demonstrated that a higher output current was not correlated with a more favourable response. Furthermore, even a low output current (<1 mA) can reduce seizure frequency in a substantial portion of patients (Bunch, et al., 2007). Moreover, we did not adjust the duty cycle to rapid cycling, that is, a mode of stimulation with a faster intermittent pulse stimulation. We presume that this did not influence seizure reduction significantly because several studies have shown that rapid cycling does not provide any additional persistent seizure control over normal cycling (Helmers, et al., 2001, Labar, 2004, Scherrmann, et al., 2001). We cannot exclude the possibility that a longer follow-up would have yielded different results. Some studies have suggested that a

larger percentage of patients respond at longer follow-up (Ben-Menachem, 2002, George, et al., 1994, Zamponi, et al.), while others do not demonstrate an effect of longer stimulation (Danielsson, et al., 2008, Majoie, et al., 2005). However in our study, the number of responders at the end of the add-on phase was not significantly different from that in the blinded phase.
An increase in seizure severity in response to VNS was observed as well, just as described in the adult trials (Ben-Menachem, et al., 1994, The VNS Study Group, 1995) and in two prospective and one retrospective paediatric trial (Helmers, et al., 2001, Hornig, et al., 1997, Parker, et al., 1999). This might be explained by the natural course of the disease, which is variable over time: the 2 patients described by Parker who experienced an initial increase in seizure frequency, both returned to baseline seizure frequencies when follow-up duration was prolonged (Parker, et al., 1999addendum). In 1 out of 6 patients described by Helmers et al. who experienced a more than 50% increase in seizure frequency after 3 months, the increased seizure frequency persisted after 6 months (Helmers, et al., 2001).

Several other studies have tried to identify a profile of responders. In line with the results of Patwardhan et al. we found a trend towards a correlation between age at onset and response to VNS. According to Jansky et al. the absence of bilateral interictal epileptic discharges (IED) and the presence of malformation of cortical development (MCD) were factors predicting a favourable outcome (Janszky, et al., 2005). In our responding group seven out of nine patients in whom 50% or more seizure frequency reduction was achieved had bilateral IED versus 18 out of 25 non-responders. Only one of the patients with MCD (n=8) had a greater than 50% seizure frequency reduction. Callosotomy prior to VNS treatment was reported to be associated with a positive response (Helmers, et al., 2001, Janszky, et al., 2005), but our patient who had undergone a callosotomy did not respond favourable.

Finally, it is important to realize that a moderate effect on seizure frequency does not automatically mean that VNS is not a suitable treatment option for children. After all, this population of patients is highly refractory. For comparison: only 3% of children with refractory epilepsy become seizure free after addition of merely a third AED (Sheth and Stafstrom, 2002). Furthermore, in contrast to the effect of AEDs, when children respond positively to VNS, this response is long-lasting (Arhan, et al., 2011, Helmers, et al., 2001, Murphy, 1999, Shahwan, et al., 2009). The benefits therefore might be of great value for the individual child with therapy-resistant epilepsy. This is especially true when the safety of the implantation procedure and favourable side effect profile of VNS are taken into account. Moreover, irrespective of the (lack of) effect on seizure frequency, the possible reduction in seizure severity and improvement of well-being makes this treatment worthwhile considering (Aldenkamp, et al., 2001, Murphy, et al., 2003).

References

Aldenkamp, A. P., Majoie, H. J., Berfelo, M. W., Evers, S. M., Kessels, A. G., Renier, W. O., and Wilmink, J., 2002. Long-term effects of 24-month treatment with vagus nerve stimulation on behaviour in children with Lennox-Gastaut syndrome. Epilepsy Behav 3**,** 475-479.

Aldenkamp, A. P., Van de Veerdonk, S. H., Majoie, H. J., Berfelo, M. W., Evers, S. M., Kessels, A. G., Renier, W. O., and Wilmink, J., 2001. Effects of 6 Months of Treatment with Vagus Nerve Stimulation on Behavior in Children with Lennox-Gastaut Syndrome in an Open Clinical and Nonrandomized Study. Epilepsy Behav 2**,** 343-350.

Alexopoulos, A. V., Kotagal, P., Loddenkemper, T., Hammel, J., and Bingaman, W. E., 2006. Long-term results with vagus nerve stimulation in children with pharmacoresistant epilepsy. Seizure 15**,** 491-503.

Arhan, E., Serdaroglu, A., Kurt, G., Bilir, E., Durdag, E., Erdem, A., Aksakal, F. N., Ozcelik, A., and Baykaner, K., 2011. The efficacy of vagal nerve stimulation in children with pharmacoresistant epilepsy: practical experience at a Turkish tertiary referral center. Eur J Paediatr Neurol 14**,** 334-339.

Arthur, T. M., Saneto, R. P., de Menezes, M. S., Devinsky, O., Lajoie, J., Murphy, P. J., Cook, W. B., and Ojemann, J. G., 2007. Vagus nerve stimulation in children with mitochondrial electron transport chain deficiencies. Mitochondrion 7**,** 279-283.

Beery, K., and Beery, N., 2006. The Beery---Buktenica Developmental Test of Visual-Motor Integration. BeeryTM VMI. Administration, Scoring and Teaching Manual. NCS Pearson, Minneapolis.

Ben-Menachem, E., 2002. Vagus-nerve stimulation for the treatment of epilepsy. Lancet Neurol 1**,** 477-482.

Ben-Menachem, E., Manon-Espaillat, R., Ristanovic, R., Wilder, B. J., Stefan, H., Mirza, W., Tarver, W. B., and Wernicke, J. F., 1994. Vagus nerve stimulation for treatment of partial seizures: 1. A controlled study of effect on seizures. First International Vagus Nerve Stimulation Study Group. Epilepsia 35**,** 616-626.

Benifla, M., Rutka, J. T., Logan, W., and Donner, E. J., 2006. Vagal nerve stimulation for refractory epilepsy in children: indications and experience at The Hospital for Sick Children. Childs Nerv Syst 22**,** 1018-1026.

Blount, J. P., Tubbs, R. S., Kankirawatana, P., Kiel, S., Knowlton, R., Grabb, P. A., and Bebin, M., 2006. Vagus nerve stimulation in children less than 5 years old. Childs Nerv Syst 22**,** 1167-1169.

Bunch, S., DeGiorgio, C. M., Krahl, S., Britton, J., Green, P., Lancman, M., Murphy, J., Olejniczak, P., Shih, J., and Heck, C. N., 2007. Vagus nerve stimulation for epilepsy: is output current correlated with acute response? Acta Neurol Scand 116**,** 217-220.

Commission on Classification and Terminology of the International League Against Epilepsy, 1989. Proposal for revised classification of epilepsies and epileptic syndromes. . Epilepsia 30**,** 389-399.

Danielsson, S., Viggedal, G., Gillberg, C., and Olsson, I., 2008. Lack of effects of vagus nerve stimulation on drug-resistant epilepsy in eight pediatric patients with autism spectrum disorders: a prospective 2-year follow-up study. Epilepsy Behav 12**,** 298-304.

De Herdt, V., Boon, P., Ceulemans, B., Hauman, H., Lagae, L., Legros, B., Sadzot, B., Van Bogaert, P., van Rijckevorsel, K., Verhelst, H., and Vonck, K., 2007. Vagus nerve stimulation for refractory epilepsy: a Belgian multicenter study. Eur J Paediatr Neurol 11**,** 261-269.

Dunn, L., and Dunn, L., 2005. Peabody Picture Vocabulary Test--III--NL, Nederlandse versie door Liesbeth Schlichting. Harcourt Assessment B.V., Amsterdam.

George, R., Salinsky, M., Kuzniecky, R., Rosenfeld, W., Bergen, D., Tarver, W. B., and Wernicke, J. F., 1994. Vagus nerve stimulation for treatment of partial seizures: 3. Long-term follow-up on first 67

patients exiting a controlled study. First International Vagus Nerve Stimulation Study Group. Epilepsia 35, 637-643.

Hallbook, T., Lundgren, J., Blennow, G., Stromblad, L. G., and Rosen, I., 2005. Long term effects on epileptiform activity with vagus nerve stimulation in children. Seizure 14, 527-533.

Handforth, A., DeGiorgio, C. M., Schachter, S. C., Uthman, B. M., Naritoku, D. K., Tecoma, E. S., Henry, T. R., Collins, S. D., Vaughn, B. V., Gilmartin, R. C., Labar, D. R., Morris, G. L., 3rd, Salinsky, M. C., Osorio, I., Ristanovic, R. K., Labiner, D. M., Jones, J. C., Murphy, J. V., Ney, G. C., and Wheless, J. W., 1998. Vagus nerve stimulation therapy for partial-onset seizures: a randomized active-control trial. Neurology 51, 48-55.

Heck, C., Helmers, S. L., and DeGiorgio, C. M., 2002. Vagus nerve stimulation therapy, epilepsy, and device parameters: scientific basis and recommendations for use. Neurology 59, S31-37.

Helmers, S. L., Wheless, J. W., Frost, M., Gates, J., Levisohn, P., Tardo, C., Conry, J. A., Yalnizoglu, D., and Madsen, J. R., 2001. Vagus nerve stimulation therapy in pediatric patients with refractory epilepsy: retrospective study. J Child Neurol 16, 843-848.

Hornig, G. W., Murphy, J. V., Schallert, G., and Tilton, C., 1997. Left vagus nerve stimulation in children with refractory epilepsy: an update. South Med J 90, 484-488.

Janszky, J., Hoppe, M., Behne, F., Tuxhorn, I., Pannek, H. W., and Ebner, A., 2005. Vagus nerve stimulation: predictors of seizure freedom. J Neurol Neurosurg Psychiatry 76, 384-389.

Kabir, S. M., Rajaraman, C., Rittey, C., Zaki, H. S., Kemeny, A. A., and McMullan, J., 2009. Vagus nerve stimulation in children with intractable epilepsy: indications, complications and outcome. Childs Nerv Syst 25, 1097-1100.

Kang, H. C., Hwang, Y. S., Kim, D. S., and Kim, H. D., 2006. Vagus nerve stimulation in pediatric intractable epilepsy: a Korean bicentric study. Acta Neurochir Suppl 99, 93-96.

Khurana, D. S., Reumann, M., Hobdell, E. F., Neff, S., Valencia, I., Legido, A., and Kothare, S. V., 2007. Vagus nerve stimulation in children with refractory epilepsy: unusual complications and relationship to sleep-disordered breathing. Childs Nerv Syst 23, 1309-1312.

Koo, B., Ham, S. D., Sood, S., and Tarver, B., 2001. Human vagus nerve electrophysiology: a guide to vagus nerve stimulation parameters. J Clin Neurophysiol 18, 429-433.

Kossoff, E. H., and Pyzik, P. L., 2004. Improvement in alertness and behavior in children treated with combination topiramate and vagus nerve stimulation. Epilepsy Behav 5, 256-259.

Labar, D., 2004. Vagus nerve stimulation for 1 year in 269 patients on unchanged antiepileptic drugs. Seizure 13, 392-398.

Lundgren, J., Amark, P., Blennow, G., Stromblad, L. G., and Wallstedt, L., 1998. Vagus nerve stimulation in 16 children with refractory epilepsy. Epilepsia 39, 809-813.

Majoie, H. J., Berfelo, M. W., Aldenkamp, A. P., Evers, S. M., Kessels, A. G., and Renier, W. O., 2001. Vagus nerve stimulation in children with therapy-resistant epilepsy diagnosed as Lennox-Gastaut syndrome: clinical results, neuropsychological effects, and cost-effectiveness. J Clin Neurophysiol 18, 419-428.

Majoie, H. J., Berfelo, M. W., Aldenkamp, A. P., Renier, W. O., and Kessels, A. G., 2005. Vagus nerve stimulation in patients with catastrophic childhood epilepsy, a 2-year follow-up study. Seizure 14, 10-18.

Murphy, J. V., 1999. Left vagal nerve stimulation in children with medically refractory epilepsy. The Pediatric VNS Study Group. J Pediatr 134, 563-566.

Murphy, J. V., Hornig, G., and Schallert, G., 1995. Left vagal nerve stimulation in children with refractory epilepsy. Preliminary observations. Arch Neurol 52, 886-889.

Murphy, J. V., Torkelson, R., Dowler, I., Simon, S., and Hudson, S., 2003. Vagal nerve stimulation in refractory epilepsy: the first 100 patients receiving vagal nerve stimulation at a pediatric epilepsy center. Arch Pediatr Adolesc Med 157**,** 560-564.

Murphy, J. V., Wheless, J. W., and Schmoll, C. M., 2000. Left vagal nerve stimulation in six patients with hypothalamic hamartomas. Pediatr Neurol 23**,** 167-168.

Nagarajan, L., Walsh, P., Gregory, P., and Lee, M., 2002. VNS therapy in clinical practice in children with refractory epilepsy. Acta Neurol Scand 105**,** 13-17.

O'Donoghue, M. F., Duncan, J. S., and Sander, J. W., 1996. The National Hospital Seizure Severity Scale: a further development of the Chalfont Seizure Severity Scale. Epilepsia 37**,** 563-571.

Parain, D., Penniello, M. J., Berquen, P., Delangre, T., Billard, C., and Murphy, J. V., 2001. Vagal nerve stimulation in tuberous sclerosis complex patients. Pediatr Neurol 25**,** 213-216.

Parker, A. P., Polkey, C. E., Binnie, C. D., Madigan, C., Ferrie, C. D., and Robinson, R. O., 1999. Vagal nerve stimulation in epileptic encephalopathies. Pediatrics 103**,** 778-782.

Patwardhan, R. V., Stong, B., Bebin, E. M., Mathisen, J., and Grabb, P. A., 2000. Efficacy of vagal nerve stimulation in children with medically refractory epilepsy. Neurosurgery 47**,** 1353-1357; discussion 1357-1358.

Rheims, S., Cucherat, M., Arzimanoglou, A., and Ryvlin, P., 2008. Greater response to placebo in children than in adults: a systematic review and meta-analysis in drug-resistant partial epilepsy. PLoS Med 5**,** e166.

Rossignol, E., Lortie, A., Thomas, T., Bouthiller, A., Scavarda, D., Mercier, C., and Carmant, L., 2009. Vagus nerve stimulation in pediatric epileptic syndromes. Seizure 18**,** 34-37.

Rychlicki, F., Zamponi, N., Cesaroni, E., Corpaci, L., Trignani, R., Ducati, A., and Scerrati, M., 2006. Complications of vagal nerve stimulation for epilepsy in children. Neurosurg Rev 29**,** 103-107.

Saneto, R. P., Sotero de Menezes, M. A., Ojemann, J. G., Bournival, B. D., Murphy, P. J., Cook, W. B., Avellino, A. M., and Ellenbogen, R. G., 2006. Vagus nerve stimulation for intractable seizures in children. Pediatr Neurol 35**,** 323-326.

Scherrmann, J., Hoppe, C., Kral, T., Schramm, J., and Elger, C. E., 2001. Vagus nerve stimulation: clinical experience in a large patient series. J Clin Neurophysiol 18**,** 408-414.

Shahwan, A., Bailey, C., Maxiner, W., and Harvey, A. S., 2009. Vagus nerve stimulation for refractory epilepsy in children: More to VNS than seizure frequency reduction. Epilepsia 50**,** 1220-1228.

Sheth, R. D., and Stafstrom, C. E., 2002. Intractable pediatric epilepsy: vagal nerve stimulation and the ketogenic diet. Neurol Clin 20**,** 1183-1194.

The VNS Study Group, 1995. A randomized controlled trial of chronic vagus nerve stimulation for treatment of medically intractable seizures. Neurology 45**,** 224-230.

Tubbs, R. S., Patwardhan, R., Palmer, C. A., Kelly, D. R., Elton, S., Blount, J. P., Bebin, M., and Grabb, P. A., 2001. Histological appearance of a chronically stimulated vagus nerve in a pediatric patient. Pediatr Neurosurg 35**,** 99-102.

Zamponi, N., Passamonti, C., Cappanera, S., and Petrelli, C., 2011. Clinical course of young patients with Dravet syndrome after vagal nerve stimulation. Eur J Paediatr Neurol 15, 8-14.

Chapter 7

The effects of vagus nerve stimulation on pro- and anti-inflammatory cytokines in children with refractory epilepsy: an exploratory study

M. Aalbers, S. Klinkenberg, K. Rijkers, P. Verschuure, A. Aldenkamp, J. Vles, M. Majoie

Adapted from Neuroimmunomodulation 2012;19:352-358

Abstract

The vagus nerve has important immunological and anti-inflammatory actions that might be relevant to the beneficial effects of vagus nerve stimulation (VNS). Therefore, we conducted an exploratory study on VNS effects on cytokine levels in plasma and cerebrospinal fluid of children suffering from refractory epilepsy. Moreover, as predictors of the response are lacking, we also aimed to determine if cytokine changes predicted the clinical response.

VNS was performed according to a randomized double-blind design: plasma levels were compared between patients who received 20 weeks of high output (therapeutic) (n=21) or low output (active control) stimulation (n=20). Thereupon, all patients received high output stimulation for another 19 weeks; levels during this period were compared to baseline. Interictal interleukin-1β, interleukin-6, and interleukin-10 were determined by ELISA.

No significant changes were found between high and low output groups and between the last nineteen weeks of stimulation and baseline. Changes in interleukin-1β correlated with improved IQ (τ=0.42, $p<0.01$). Lower baseline plasma levels of interleukin-6 were associated with more seizure frequency reduction (R^2=0.105(1,35), p=0.050).

In conclusion, interictal cytokine levels were not altered by VNS but baseline interleukin-6 predicted the clinical response. In the future patient selection may be aided by determination of the cytokine profile of the patient.

Introduction

Vagus nerve stimulation (VNS) is an adjunctive treatment for medically refractory epilepsy. In addition to anticonvulsive effects, VNS has positive effects on behavior, mood, and cognition (Schachter, 2004). Not all patients respond to VNS: in epilepsy, 23-39% of VNS treated patients experience more than 50% seizure frequency reduction versus 13-19% of placebo-treated patients who received non-therapeutic low output stimulation (Ben-Menachem, et al., 1994, Handforth, et al., 1998, The Vagus Nerve Stimulation Study Group, 1995). It is unclear why the effects of VNS are limited and predictors to identify patients that are most likely to respond are lacking. Moreover, the mechanism of action of VNS is not fully understood.

In this context, the immunomodulatory function of the vagus nerve is of particular interest. Afferent signals can activate the so-called cholinergic anti-inflammatory pathway upon inflammation (Pavlov and Tracey, 2005). Through this pathway, efferent vagus nerve fibers inhibit the release of pro-inflammatory cytokines and in this way reduce inflammation. In recent years, inflammation has been strongly implicated in the development of seizures and epilepsy (Vezzani, et al., 2011) and therefore the activation of the anti-inflammatory pathway by VNS could decrease the inflammatory response and thereby explain its clinical effects.

While several animal studies have demonstrated that electrical and chemical stimulation of the vagus nerve affects the immune response (Bernik, et al., 2002, Borovikova, et al., 2000, Guarini, et al., 2003, Guarini, et al., 2004, Hosoi, et al., 2000, Huston, et al., 2007, Luyer, et al., 2005, Wang, et al., 2004, Wang, et al., 2003, Wu, et al., 2007), only a few studies on the immunomodulatory effects of VNS have been performed in humans. VNS has been reported to increase circulating pro- and anti-inflammatory cytokines in depressed patients (Corcoran, et al., 2005), but in epilepsy patients the results are inconclusive: VNS has been shown to alter the immune response (De Herdt, et al., 2009) but fails to affect systemic inflammation (Barone, et al., 2007, Majoie, et al., 2011). So far there are no studies available evaluating neuroimmunomodulation by VNS in children. Therefore the purpose of this study was to determine whether VNS treatment caused changes in cytokine levels in children and if these changes were related to the clinical effects like seizure frequency reduction and change in IQ. Furthermore, we aimed to determine if the changes in cytokine levels could predict clinical response. Therefore, we determined levels of interleukin (IL)-1β and IL-6, while these cytokines were increased in epilepsy patients (Boer, et al., 2008, Choi, et al., 2009, Ravizza, et al., 2008, Shu, et al., 2010, Varella, et al., 2011, Yu, et al., 2012). Additionally, we also evaluated levels of IL-10, as this is one of the most important anti-inflammatory cytokines (Sabat, et al., 2010).

Materials and methods

Patients

We included 41 children (23 boys) with medically refractory epilepsy, which was defined as failure of adequate trials of at least two tolerated, appropriately chosen and used antiepileptic drug (AED) schedules to achieve sustained seizure freedom (Kwan, et al., 2010). The mean age at implantation was 11.2 years. Thirty-five patients had localization related epilepsy and 6 patients had generalized epilepsy (for details see Table 7.1). Twenty patients had focal seizures, 11 patients had generalized seizures, and 10 patients had both focal and generalized seizures. All patients had undergone an extensive clinical evaluation, which included EEG, CT/MRI, and genetic and/ or metabolic screening as needed. Patients with a progressive cerebral lesion, malignancy, or any degenerative or immunological disorder such as Rasmussen encephalitis or acute disseminated encephalomyelitis were excluded. Medication regimens remained unchanged throughout the study and none of the patients were treated with antihistamines, metoclopramide, or anti-inflammatory drugs. Visits were postponed if patients displayed any clinical signs of infection during the study. The most frequent administered AEDs included valproic acid (n=18), lamotrigine (n=16), clobazam (n=11), topiramate (n=10), and levetiracetam (n=9).

Table 7.1 Patient characteristics.

	High output VNS	Low output VNS
Number of patients (male/ female)	21 (11/10)	20 (12/8)
Mean age at implantation (range)	10y11m (3y10m-17y8m)	11y6m (4y2m-17y2m)
Mean age at onset (range)	2y10m (0-12)	1y8m (0-5)
Mean interval onset-implantation (range)	7y8m (2-16)	9y5m (3-15)
Median seizure frequency at baseline (range), seizures/day	2.1 (0.1-53.7)	0.9 (0.1-31.7)
ILAE classification (Epilepsy, 1989)		
Localization related	19 (90%)	16 (80%)
Symptomatic	15 (71%)	10 (50%)
Cryptogenic	4 (19%)	6 (30%)
Generalized	2 (10%)	4 (20%)
Idiopathic	0	2 (10%)
Symptomatic	2 (10%)	2 (10%)
Total number of AEDs ever administered (range)	7.0 (5-10)	7.3 (4-14)
Mean number of AEDs used during the study (range)	2.5 (1-5)	2.5 (1-4)
Mean maximal output during blinded phase, mA	0.99	n.a.
Mean maximal output during add-on phase, mA	1.98	1.29

M= months; n.a.= not applicable; y= years.

After a baseline period of 12 weeks, patients were implanted with a programmable pulse generator (Neurocybernetic prothesis NCP, Cyberonics Inc., Webster, TX, USA). Patients were randomly assigned to two groups: patients received either high (therapeutic) or low (active control) stimulation. This active control group was

incorporated to protect the blinding, because patients can feel the stimulation, and to control for the presence of the electrode. Initial stimulation parameters are depicted in Table 7.2. In the treatment group, current was stepwise increased with 2-week intervals to the maximally tolerated output current (maximum 1.75 mA). After 20 weeks, all patients entered an non-blinded follow-up of 19 weeks. During this add-on phase both groups received high output stimulation (maximum 2.25 mA) (see Figure 7.1 for study design).
All procedures were approved by the Central Medical Ethical Committee of Maastricht University Medical Centre and written informed consent was obtained from all caregivers and patients if aged over 12.

Table 7.2 Initial device settings.

	Active control group	Treatment group
Output current (mA)	0.25	0.25
Pulse width (ms)	0.1	0.5
Frequency (Hz)	1	30
Duty cycle: on (sec)/ off (min)	14/60	30/5

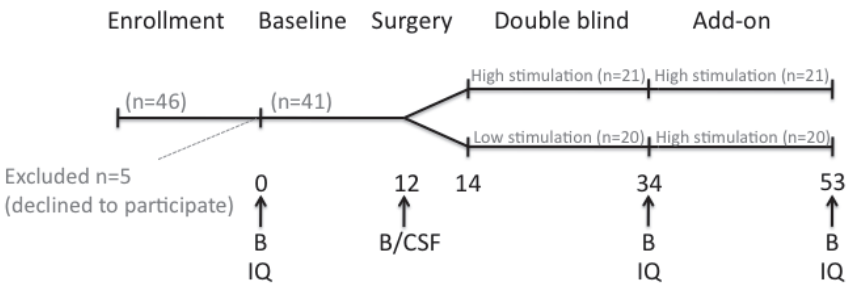


Figure 7.1 Study design.
After a 12-week baseline, patients were implanted with the vagus nerve stimulator. During the double blind phase (20 weeks) half of the patients received high output stimulation and the other half received low output stimulation. During the add-on phase (19 weeks), all patients received high output stimulation. Arrows indicate data and sample collection: B= blood, CSF= cerebro spinal fluid; time depicted in weeks.

VNS effects

We evaluated VNS effects on seizure frequency and cognitive function. Seizure frequency was recorded in a seizure diary by caregivers. The effect of VNS was calculated by expressing the mean seizure frequency during the last 19 weeks of VNS as a percentage of the seizure frequency at baseline.

Cognitive function was assessed using the Peabody Picture Vocabulary test (PPVT-III-NL) (Dunn and Dunn, 2005) and the Beery VMI 5th edition (Beery and Beery, 2006). To calculate IQ, raw scores were converted to age-equivalents in months, which were divided by the age in months at the date of testing and multiplied by 100. Finally, the average of both tests was calculated.

Cytokine measurements

EDTA plasma was obtained at baseline, at the end of the blinded phase after 20 weeks of stimulation, and at the end of the add-on phase after 39 weeks of stimulation (Figure 7.1). Plasma and cerebrospinal fluid (CSF) were also collected immediately before surgery when patients were under general anaesthesia. Patients did not experience seizures in more than 12 hours preceding sample collection in order to determine interictal, overall cytokine levels.
Levels of IL-1β, IL-6, and IL-10 were determined in duplicate using commercially available enzyme-linked immunosorbent assays (R&D Systems, Abingdon, UK; detection limits 0.125, 0.156, and 0.78 pg/ml respectively) following the manufacturer's instructions. Values below the detection limit were converted to a value of 0.5 times the detection limit for statistical analysis, as these cannot be considered missing (Majoie, et al., 2011, Ricker, et al., 2011).

Statistics

Statistical analysis was performed using SPSSv19 for MacOSX. Cytokine levels at the end of the blinded phase were compared between the high and low output groups using the Mann-Whitney U Test. Cytokine levels at the end of the add-on phase were compared with baseline by a related-samples Wilcoxon signed-rank test.
To analyze the relationship between cytokines changes and VNS effects we performed a correlation analysis (Kendall's tau) between the percentage change of cytokines and IQ and seizure frequency.
To evaluate whether baseline and pre-operative cytokine plasma and CSF levels could predict seizure frequency reduction, univariate linear regression analysis was performed using seizure reduction during the add-on phase as the dependent variable.
A p-value <0.05 was considered statistically significant.

Results

There were no statistically significant differences in cytokine levels between the high and low stimulation group at the end of the blinded phase or between cytokine levels at baseline and at the end of the add-on phase (Table 7.3).

Table 7.3 Plasma cytokine levels.

	Baseline	Blind phase: active control group	Blind phase: treatment group	End of add-on	P[1]	P[2]
IL-1β (pg/ml)	0.06 (0.06-8.00)	0.14 (0.06-1.30)	0.06 (0.06-0.62)	0.14 (0.06-1.92)	0.87	0.73
IL-6 (pg/ml)	1.16 (0.23-9.57)	1.09 (0.25-17.39)	1.27 (0.30-10.13)	1.08 (0.37-18.57)	0.64	0.79
IL-10 (pg/ml)	0.78 (0.39-6.87)	0.78 (0.39-5.46)	0.78 (0.39-7.53)	0.78 (0.39-14.58)	0.50	0.10

Values are medians (range); [1]Comparison of active control and treatment group by Mann Whitney U test; [2]Comparison of levels at baseline and at the end of the add-on phase by related-samples Wilcoxon signed rank test.

Seizure frequency reduction and cytokine changes

Four patients were excluded from seizure frequency analysis due to incomplete seizure diaries. Twenty-two percent (n=8) of the patients experienced ≥50% seizure frequency reduction. The change in seizure frequency during the add-on phase did not correlate with changes in cytokine levels.

Cognitive function and cytokine changes

Fourteen patients could not participate in cognitive testing due to cognitive and/or physical limitations. The IQ tended to increase from a mean score of 55.1 (baseline) to 58.2 (end of the add-on phase) (p=0.08, paired t-test). The effects on cognition were positively correlated with a change in IL-1β (τ=.42, p<0.01): the larger the IL-1β increase, the greater the improvement of IQ. Changes in IL-6 and IL-10 did not significantly correlate with changes in IQ.

Predictive value of baseline cytokines

Baseline plasma levels of IL-6 predicted seizure frequency reduction during the add-on phase (R^2=.105(1,35), p=0.050): lower IL-6 levels were associated with more seizure frequency reduction. Pre-implantation CSF levels did not predict seizure frequency reduction.

Discussion

To our knowledge, this exploratory study is the first study to evaluate the effects of VNS on cytokine levels in children. VNS reduced seizure frequency by more than 50% in 22% of the patients. We were unable to confirm our hypothesis that VNS evokes an anti-inflammatory reaction since we did not find a statistically significant effect of VNS on interictal plasma cytokine levels. Similar to our results, two studies in adults suffering from medically refractory epilepsy did not show significant changes in blood levels of IL-6, IL-10, and tumor necrosis factor-α (TNF-α) (Barone, et al., 2007, Majoie, et al., 2011). A study in adult depressed patients in contrast showed that 3 months of

VNS increased plasma levels of IL-6, TNF-α, and transforming growth factor-β (Corcoran, et al., 2005).

Several reasons might explain the absence of the hypothesized VNS-induced anti-inflammatory reaction. Firstly, VNS did not alter interictal cytokine levels. Since epilepsy patients are known to have altered interictal cytokine levels (Hulkkonen, et al., 2004, Liimatainen, et al., 2009, Nowak, et al., 2011), we specifically determined whether VNS changed cytokine levels more than 12 h after a seizure. Nevertheless although these cytokine levels were unchanged, we cannot exclude the possibility that VNS treatment influences cytokine levels at a different time point. For example, the chosen interval might not have been sufficient for cytokines to return to their baseline levels. On the other hand, if VNS only inhibits cytokine expression in the immediate post-ictal phase this effect would not be detected using the present study design. Although technically challenging, future studies should compare cytokine levels immediately before and after a seizure (Alapirtti, et al., 2009, Lehtimaki, et al., 2007).

Furthermore, although VNS did not alter plasma cytokine levels, it may still have affected cytokine levels within the central nervous system (CNS). CSF cytokine levels are probably the best representation of CNS cytokine levels available. Because ethical reasons limit CSF collection in children, it was only obtained at one time point, which made longitudinal monitoring impossible.

There are several limitations that might account for the lack of changes in cytokine levels. For example, the study included a relatively small number of patients who suffered from different epilepsy syndromes. This might have limited the possibility of detecting significant effects as there is evidence that plasma cytokine concentrations differ between various etiologies and pathologies (Alapirtti, et al., 2009, Bauer, et al., 2009, Liimatainen, et al., 2009). In addition, the relatively low responder rate may have affected an association between the anticonvulsive effectiveness of VNS and any cytokine changes.

Contrary to seizure frequency reduction, changes in IQ were correlated with altered levels of IL-1β: a larger increase in IL-1β was associated with a larger improvement of IQ. IL-1β is a pro-inflammatory cytokine, which has proconvulsive properties (Vezzani, et al., 1999). It is also implicated in cognitive function and has been shown to have both beneficial and detrimental effects on learning and memory (Huang and Sheng, 2010). However, as no causality can be inferred from the current analysis, future studies should explore whether changes in IL-1β actually contribute to improved cognition upon VNS.

Our study revealed that low baseline IL-6 levels correspond to a greater reduction in seizure frequency and thus predict the clinical response. IL-6 is a cytokine with both pro- and anti-inflammatory characteristics that mediates the acute phase reaction and fever. The predictive value of IL-6 is particularly interesting since IL-6 is the most consistently and chronically changed circulating cytokine in epilepsy (Hulkkonen, et al., 2004, Liimatainen, et al., 2009, Nowak, et al., 2011). However, other investigators failed to detect any cytokine changes that were of potential predictive value (De Herdt,

et al., 2009), possibly due to the fact that the changes were evaluated in epileptic adults using whole blood samples that were stimulated with lipopolysaccharide. In addition, studies in adults cannot be compared to studies in children without taking into account the age dependency of cytokine availability (Straub, et al., 2001).

Conclusion

VNS decreases seizure frequency by more than 50% in 22% of paediatric epilepsy patients and may improve IQ. Larger increases in IL-1β are related to a greater improvement of IQ. Low baseline IL-6 levels predict a greater reduction in seizure frequency. Thus the determination of IL-6 prior to VNS treatment may be a valuable tool to aid responder identification in future.

References

Alapirtti, T., Rinta, S., Hulkkonen, J., Makinen, R., Keranen, T., and Peltola, J., 2009. Interleukin-6, interleukin-1 receptor antagonist and interleukin-1beta production in patients with focal epilepsy: A video-EEG study. J Neurol Sci 280**,** 94-97.

Barone, L., Colicchio, G., Policicchio, D., Di Clemente, F., Di Monaco, A., Meglio, M., Lanza, G. A., and Crea, F., 2007. Effect of vagal nerve stimulation on systemic inflammation and cardiac autonomic function in patients with refractory epilepsy. Neuroimmunomodulation 14**,** 331-336.

Bauer, S., Cepok, S., Todorova-Rudolph, A., Nowak, M., Koller, M., Lorenz, R., Oertel, W. H., Rosenow, F., Hemmer, B., and Hamer, H. M., 2009. Etiology and site of temporal lobe epilepsy influence postictal cytokine release. Epilepsy Res 86**,** 82-88.

Beery, K., and Beery, N., 2006. The Beery-Buktenica Developmental Test of Visual-Motor Integration. BeeryTM VMI. Administration, Scoring and Teaching Manual. NCS Pearson, Minneapolis.

Ben-Menachem, E., Manon-Espaillat, R., Ristanovic, R., Wilder, B. J., Stefan, H., Mirza, W., Tarver, W. B., and Wernicke, J. F., 1994. Vagus nerve stimulation for treatment of partial seizures: 1. A controlled study of effect on seizures. First International Vagus Nerve Stimulation Study Group. Epilepsia 35**,** 616-626.

Bernik, T. R., Friedman, S. G., Ochani, M., DiRaimo, R., Susarla, S., Czura, C. J., and Tracey, K. J., 2002. Cholinergic antiinflammatory pathway inhibition of tumor necrosis factor during ischemia reperfusion. J Vasc Surg 36**,** 1231-1236.

Boer, K., Jansen, F., Nellist, M., Redeker, S., van den Ouweland, A. M., Spliet, W. G., van Nieuwenhuizen, O., Troost, D., Crino, P. B., and Aronica, E., 2008. Inflammatory processes in cortical tubers and subependymal giant cell tumors of tuberous sclerosis complex. Epilepsy Res 78**,** 7-21.

Borovikova, L. V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G. I., Watkins, L. R., Wang, H., Abumrad, N., Eaton, J. W., and Tracey, K. J., 2000. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405**,** 458-462.

Choi, J., Nordli, D. R. Jr., Alden, T. D., DiPatri, A., Jr., Laux, L., Kelley, K., Rosenow, J., Schuele, S. U., Rajaram, V., and Koh, S., 2009. Cellular injury and neuroinflammation in children with chronic intractable epilepsy. J Neuroinflammation 6**,** 38.

Commission on Classification and Terminology of the International League Against Epilepsy, 1989. Proposal for revised classification of epilepsies and epileptic syndromes. . Epilepsia 30**,** 389-399.

Corcoran, C., Connor, T. J., O'Keane, V., and Garland, M. R., 2005. The effects of vagus nerve stimulation on pro- and anti-inflammatory cytokines in humans: a preliminary report. Neuroimmunomodulation 12**,** 307-309.

De Herdt, V., Bogaert, S., Bracke, K. R., Raedt, R., De Vos, M., Vonck, K., and Boon, P., 2009. Effects of vagus nerve stimulation on pro- and anti-inflammatory cytokine induction in patients with refractory epilepsy. J Neuroimmunol 214**,** 104-108.

Dunn, L., and Dunn, L., 2005. Peabody Picture Vocabulary Test--III--NL, Nederlandse versie door Liesbeth Schlichting. Harcourt Assessment B.V., Amsterdam.

Guarini, S., Altavilla, D., Cainazzo, M. M., Giuliani, D., Bigiani, A., Marini, H., Squadrito, G., Minutoli, L., Bertolini, A., Marini, R., Adamo, E. B., Venuti, F. S., and Squadrito, F., 2003. Efferent vagal fibre stimulation blunts nuclear factor-kappaB activation and protects against hypovolemic hemorrhagic shock. Circulation 107**,** 1189-1194.

Guarini, S., Cainazzo, M. M., Giuliani, D., Mioni, C., Altavilla, D., Marini, H., Bigiani, A., Ghiaroni, V., Passaniti, M., Leone, S., Bazzani, C., Caputi, A. P., Squadrito, F., and Bertolini, A., 2004. Adrenocorticotropin reverses hemorrhagic shock in anesthetized rats through the rapid activation of a vagal anti-inflammatory pathway. Cardiovasc Res 63**,** 357-365.

Handforth, A., DeGiorgio, C. M., Schachter, S. C., Uthman, B. M., Naritoku, D. K., Tecoma, E. S., Henry, T. R., Collins, S. D., Vaughn, B. V., Gilmartin, R. C., Labar, D. R., Morris, G. L., 3rd, Salinsky, M. C., Osorio, I., Ristanovic, R. K., Labiner, D. M., Jones, J. C., Murphy, J. V., Ney, G. C., and Wheless, J. W., 1998. Vagus nerve stimulation therapy for partial-onset seizures: a randomized active-control trial. Neurology 51**,** 48-55.

Hosoi, T., Okuma, Y., and Nomura, Y., 2000. Electrical stimulation of afferent vagus nerve induces IL-1beta expression in the brain and activates HPA axis. Am J Physiol Regul Integr Comp Physiol 279**,** R141-147.

Huang, Z. B., and Sheng, G. Q., 2010. Interleukin-1beta with learning and memory. Neurosci Bull 26**,** 455-468.

Hulkkonen, J., Koskikallio, E., Rainesalo, S., Keranen, T., Hurme, M., and Peltola, J., 2004. The balance of inhibitory and excitatory cytokines is differently regulated in vivo and in vitro among therapy resistant epilepsy patients. Epilepsy Res 59**,** 199-205.

Huston, J. M., Gallowitsch-Puerta, M., Ochani, M., Ochani, K., Yuan, R., Rosas-Ballina, M., Ashok, M., Goldstein, R. S., Chavan, S., Pavlov, V. A., Metz, C. N., Yang, H., Czura, C. J., Wang, H., and Tracey, K. J., 2007. Transcutaneous vagus nerve stimulation reduces serum high mobility group box 1 levels and improves survival in murine sepsis. Crit Care Med 35**,** 2762-2768.

Kwan, P., Arzimanoglou, A., Berg, A. T., Brodie, M. J., Allen Hauser, W., Mathern, G., Moshe, S. L., Perucca, E., Wiebe, S., and French, J., 2010. Definition of drug resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAE Commission on Therapeutic Strategies. Epilepsia 51**,** 1069-1077.

Lehtimaki, K., Keranen, T., Palmio, J., Makinen, R., Hurme, M., Honkaniemi, J., and Peltola, J., 2007. Increased plasma levels of cytokines after seizures in localization-related epilepsy. Acta Neurol Scand 116**,** 226-230.

Liimatainen, S., Fallah, M., Kharazmi, E., Peltola, M., and Peltola, J., 2009. Interleukin-6 levels are increased in temporal lobe epilepsy but not in extra-temporal lobe epilepsy. J Neurol 256**,** 796-802.

Luyer, M. D., Greve, J. W., Hadfoune, M., Jacobs, J. A., Dejong, C. H., and Buurman, W. A., 2005. Nutritional stimulation of cholecystokinin receptors inhibits inflammation via the vagus nerve. J Exp Med 202**,** 1023-1029.

Majoie, H. J., Rijkers, K., Berfelo, M. W., Hulsman, J. A., Myint, A., Schwarz, M., and Vles, J. S., 2011. Vagus nerve stimulation in refractory epilepsy: effects on pro- and anti-inflammatory cytokines in peripheral blood. Neuroimmunomodulation 18**,** 52-56.

Nowak, M., Bauer, S., Haag, A., Cepok, S., Todorova-Rudolph, A., Tackenberg, B., Norwood, B., Oertel, W. H., Rosenow, F., Hemmer, B., and Hamer, H. M., 2011. Interictal alterations of cytokines and leukocytes in patients with active epilepsy. Brain Behav Immun 25**,** 423-428.

Pavlov, V. A., and Tracey, K. J., 2005. The cholinergic anti-inflammatory pathway. Brain Behav Immun 19**,** 493-499.

Ravizza, T., Gagliardi, B., Noe, F., Boer, K., Aronica, E., and Vezzani, A., 2008. Innate and adaptive immunity during epileptogenesis and spontaneous seizures: evidence from experimental models and human temporal lobe epilepsy. Neurobiol Dis 29**,** 142-160.

Ricker, L. J., Kijlstra, A., Kessels, A. G., de Jager, W., Liem, A. T., Hendrikse, F., and La Heij, E. C., 2011. Interleukin and growth factor levels in subretinal fluid in rhegmatogenous retinal detachment: a case-control study. PLoS One 6**,** e19141.

Sabat, R., Grutz, G., Warszawska, K., Kirsch, S., Witte, E., Wolk, K., and Geginat, J., 2010. Biology of interleukin-10. Cytokine Growth Factor Rev 21, 331-344.

Schachter, S. C., 2004. Vagus nerve stimulation: mood and cognitive effects. Epilepsy Behav 5 Suppl 1, S56-59.

Shu, H. F., Zhang, C. Q., Yin, Q., An, N., Liu, S. Y., and Yang, H., 2010. Expression of the interleukin 6 system in cortical lesions from patients with tuberous sclerosis complex and focal cortical dysplasia type IIb. J Neuropathol Exp Neurol 69, 838-849.

Straub, R. H., Cutolo, M., Zietz, B., and Scholmerich, J., 2001. The process of aging changes the interplay of the immune, endocrine and nervous systems. Mech Ageing Dev 122, 1591-1611.

The Vagus Nerve Stimulation Study Group, 1995. A randomized controlled trial of chronic vagus nerve stimulation for treatment of medically intractable seizures. Neurology 45, 224-230.

Varella, P. P., Santiago, J. F., Carrete, H., Jr., Higa, E. M., Yacubian, E. M., Centeno, R. S., Caboclo, L. O., Castro Neto, E. F., Canzian, M., Amado, D., Cavalheiro, E. A., and Naffah-Mazzacoratti Mda, G., 2011. Relationship between fluid-attenuated inversion-recovery (FLAIR) signal intensity and inflammatory mediator's levels in the hippocampus of patients with temporal lobe epilepsy and mesial temporal sclerosis. Arq Neuropsiquiatr 69, 91-99.

Vezzani, A., Conti, M., De Luigi, A., Ravizza, T., Moneta, D., Marchesi, F., and De Simoni, M. G., 1999. Interleukin-1beta immunoreactivity and microglia are enhanced in the rat hippocampus by focal kainate application: functional evidence for enhancement of electrographic seizures. J Neurosci 19, 5054-5065.

Vezzani, A., French, J., Bartfai, T., and Baram, T. Z., 2011. The role of inflammation in epilepsy. Nat Rev Neurol 7, 31-40.

Wang, H., Liao, H., Ochani, M., Justiniani, M., Lin, X., Yang, L., Al-Abed, Y., Metz, C., Miller, E. J., Tracey, K. J., and Ulloa, L., 2004. Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. Nat Med 10, 1216-1221.

Wang, H., Yu, M., Ochani, M., Amella, C. A., Tanovic, M., Susarla, S., Li, J. H., Yang, H., Ulloa, L., Al-Abed, Y., Czura, C. J., and Tracey, K. J., 2003. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature 421, 384-388.

Wu, R., Dong, W., Cui, X., Zhou, M., Simms, H. H., Ravikumar, T. S., and Wang, P., 2007. Ghrelin down-regulates proinflammatory cytokines in sepsis through activation of the vagus nerve. Ann Surg 245, 480-486.

Yu, N., Di, Q., Hu, Y., Zhang, Y. F., Su, L. Y., Liu, X. H., and Li, L. C., 2012. A meta-analysis of pro-inflammatory cytokines in the plasma of epileptic patients with recent seizure. Neurosci Lett 514, 110-115.

Chapter 8

Animal models for vagus nerve stimulation in epilepsy

M. Aalbers, J. Vles, S. Klinkenberg, G. Hoogland, M. Majoie, K. Rijkers

Experimental Neurology 2011;230:167-75

Abstract

Vagus nerve stimulation (VNS) is a moderately effective adjunctive treatment for patients suffering from medically refractory epilepsy and is explored as a treatment option for several other disorders. The present review provides a critical appraisal of the studies on VNS in animal models of seizures and epilepsy. So far, these studies mostly applied short-term VNS in seizure models, demonstrating that VNS can suppress and prevent seizures and affect epileptogenesis. However, the mechanism of action is still largely unknown. Moreover, studies with a clinically more relevant setup where VNS is chronically applied in epilepsy models are scarce. Future directions for research and the application of this technology in animal models of epilepsy are discussed.

Introduction

Vagus nerve stimulation (VNS) is a neuromodulatory treatment that is used as an adjunctive therapy for patients with medically refractory epilepsy who are not suitable candidates for resective brain surgery or for whom surgery has failed. VNS consists of chronic intermittent electrical stimulation of the vagus nerve, delivered by a programmable pulse generator. This pulse generator is surgically implanted subcutaneously in the chest wall and connected to a bipolar electrode that is wrapped around the vagus nerve in the neck. Already in the late 19th century a primitive vagus nerve stimulator was used to treat epilepsy (Lanska, 2002). More than one century later, in 1988, the first epilepsy patient was implanted with a modern stimulating device (Penry and Dean, 1990). Since then, more than 55.000 patients have been implanted worldwide. VNS is also an adjunctive therapy for treatment resistant depression and has gained attention as a possible treatment option for several other disorders (Beekwilder and Beems, 2010).
Not all epilepsy patients respond to VNS: seizure frequency is reduced with more than 50% in approximately one third of the patients (DeGiorgio, et al., 2000). This success rate is even lower when corrected for the natural course of the disease and placebo effect.
Several studies on the effectiveness, safety, optimal stimulation parameters, and mechanism of action have been conducted both in humans and animals. The effectiveness and safety have been shown repetitively but the anticonvulsive mechanism of action has not been fully elucidated.
This review aims at providing an overview of the preclinical studies on VNS with regard to seizures and epilepsy and suggests future directions for research and the application of this technology in animal models of epilepsy.

A literature search was performed in PubMed, The Cochrane Library, and Embase using the keywords “animal” combined with “vagus nerve stimulation” or “vagal nerve stimulation” or “VNS”, and with either “seizure” or “epilepsy”. This search resulted in a total of 564, 302, and 10 hits in Pubmed, Embase, and The Cochrane Library respectively. After counting out overlap, 41 papers were selected. Citation tracking resulted in an additional 36 papers.

The vagus nerve

The vagus nerve is the tenth cranial nerve. The largest part of the nerve, 80%, consists of afferent fibres (Paintal, 1973). Ninety percent of the afferents and 70% of the efferents are unmyelinated C-fibres (Asala and Bower, 1986), while the rest of the fibres consist of myelinated A- and B-fibres that are normally involved in mediating reflexes (Paintal, 1973). Its efferent fibres that provide parasympathetic innervation to

the heart, lungs, gastro-intestinal tract, and other intra-abdominal organs originate in the visceromotor dorsal nucleus of the vagus nerve. The efferent motor neurons that innervate the striated muscles of larynx and pharynx originate in the nucleus ambiguous.
The afferent fibres carry information on visceral sensation, somatic sensation, and taste from receptors in the peripheral organs to the central nervous system. Their cell bodies are located in the superior vagal (jugular) ganglion and the inferior vagal (nodose) ganglion and they end in the nucleus of the solitary tract (NTS).
The NTS projects directly and indirectly to the parabrachial nucleus (Fulwiler and Saper, 1984, Granata and Kitai, 1989), the dorsal raphe nucleus (Aghajanian and Wang, 1977), the locus coeruleus (Van Bockstaele, et al., 1999), the hypothalamus (Ricardo and Koh, 1978), the thalamus, the amygdala (Hopkins and Holstege, 1978, Ricardo and Koh, 1978), and the hippocampus (Castle, et al., 2005). It is thought that these afferent projections are responsible for the anticonvulsive effects of VNS.

The effects of VNS

In the following part, VNS-induced EEG-changes will be summarized first, followed by the effects of acute VNS on seizures, the effects of chronic VNS on epilepsy, the antiepileptogenic effects of VNS, and its side effects.

VNS induced EEG changes

Epilepsy is characterized by specific ictal and interictal epileptiform discharges on the electroencephalogram (EEG). Therefore many studies have investigated the influence of VNS on the EEG. In 1938 Bailey and Bremer already described that stimulation of the central part of the dissected vagus nerve increased EEG potentials recorded at the frontobasal cortex of encéphale isolé cat (Bailey and Bremer, 1938). Their publication was followed by several other animal studies on VNS-induced changes in EEG describing evoked potentials and background activity. Three groups were able to *evoke potentials* by VNS in cortex, thalamus, hypothalamus, and amygdala of cats and monkeys (Dell and Olson, 1951, Dell and Olson, 1951, Juhasz, et al., 1985, MacLean, 1990, O'Brien, et al., 1971). However, MacLean and Pribram were not able to evoke potentials in cats (MacLean, 1990).
The effect of VNS on EEG *background activity* is variable: it can induce both synchronization and desynchronization, depending on the stimulation parameters used (Balzamo and Jammes, 1989, Chase, et al., 1966, Magnes, et al., 1961). Furthermore, VNS changed the EEG from low-voltage fast activity into high-voltage

slow activity or vice versa when applied in resting satiated cats (Peñaloza-Rojas, 1964, Peñaloza-Rojas, et al., 1969).

VNS induced anticonvulsive effects

Acute VNS suppresses many different seizure types

The vast majority of animal experiments comprise acute experiments in which the anti-seizure effect of VNS was tested. In these experiments the VNS pulse was administered just before, during, or after the onset of an induced seizure. A complete overview of the effects of acute VNS in animals is provided in Table 8.1. Results are classified according to the human seizures that the models represent (Pitkanen, et al., 2006).

Models for partial seizures

VNS increases the threshold for focal motor seizures in the cortical stimulation rat model (De Herdt, et al., 2010). It also diminishes or aborts complex partial seizures evoked by intrahippocampal administration of pilocarpine (Meurs, et al., 2008) and secondarily generalized seizures induced by kainic acid and topically applied strychnine and penicillin (Stoica and Tudor, 1967, Stoica and Tudor, 1968, Zanchetti, et al., 1952). However, some of the strychinine induced spikes and motor seizures were enhanced by VNS (Stoica and Tudor, 1967, Stoica and Tudor, 1968). In addition to seizures, VNS also attenuates interictal spike activity: it reduced the frequency of penicillin-induced interictal spikes by 33% (McLachlan, 1993). (See Table 8.1)

Models for generalized seizures

VNS positively affected seizures induced by low doses of pentylenetetrazol (PTZ) (Krahl, et al., 2001, Krahl, et al., 2003), a model used to screen the activity of anticonvulsive treatments in absence seizures (Pitkanen, et al., 2006). Effectiveness did not depend on stimulation side: both left and right sided stimulation were effective (Krahl, et al., 2003).
VNS furthermore reduced or abolished tonic clonic seizures induced by high doses of PTZ (Sahin, et al., 2009, Zhang, et al., 2008), mercaptopropionic acid (3-MPA) (Sunderam, et al., 2001, Woodbury and Woodbury, 1990), and maximal electroshock (Krahl, et al., 1998, Woodbury and Woodbury, 1990, Woodbury and Woodbury, 1991). The occurrence of generalized seizures induced by systemically applied strychnine is also reduced by VNS (Zabara, 1985, Zabara, 1992). (See Table 8.1)

Table 8.1 Acute VNS in seizure and epilepsy models.

	Model represents	Method of generation		Read-out parameter	VNS effect	Reference
Seizures	Focal seizure	Electrically	Stimulation motor cortex	Motor seizure threshold	↑	De Herdt (2010)
	Complex partial seizure	Chemically	Pilocarpine	Number of seizures/ seizure severeness	↓	Meurs (2008)
	Partial seizure evolving to secondarily generalized seizure	Chemically	Kainic acid	EEG amplitude	↓	Hotta (2010)
			Penicillin topically applied	Ictal discharge (frequency/ magnitude)	↓	Godlevsky (1994)
				Interictal discharge (spike frequency)	↓	McLachlan (1993)
				Seizure occurrence	↓	Yang (2007)
			Strychnine topically applied	Spiking frequency	↓	Stoica (1967, 1968) Zanchetti (1956)
				Spiking frequency	↑	Stoica (1967, 1968)
				Convulsive waves	=	Zanchetti (1956)
	Absence	Chemically	PTZ (low dose < 60 mg/kg)	Seizure severity	↓	Krahl (2001, 2003) Takaya (1996)
				Number of seizures	↓	Takaya (1996)
				Seizure duration	↓	Takaya (1996)
				Seizure duration	=	Takaya (1996) Woodbury (1990)
	Tonic clonic	Chemically	PTZ (high dose > 60 mg/kg)	Generalized component seizure	↓	Sahin (2009)
				Seizure severity	↓	Sahin (2009)
				Inhibition field potentials	39-50%	Zhang (2008)
			3-MPA	Latency to first seizure	↑	Sunderam (2001)
				Seizure duration	↓	Sunderam (2001) Woodbury (1990)
		Electrically	Maximal electroshock	Seizure duration	↓	Krahl (1998), Woodbury (1990, 1991)
	Other	Chemically	Strychnine systemically applied	Seizure occurrence	↓	Zabara (1985, 1992)
Epilepsy	Temporal lobe epilepsy	Electrically	Kindling	After discharge duration	↓	Dedeurwaerdere (2006)
				Seizure duration	↓	Naritoku (1996), Rijkers (2009)
				Seizure severity	=	Naritoku (1996), Raedt (2009)
				Latency to generalized seizure	↑	Rijkers (2009)
	Absence epilepsy	Genetically	GAERS	Duration spike wave discharge	↑/=	Dedeurwaerdere (2004,2005)

↑ increase; ↓decrease; =no difference; PTZ=phenylpenetetrazol; 3-MPA=3-mercaptopropanoic acid; EEG=electroencephalogram.

Variable effects of acute VNS in chronic epilepsy models

Models for absence epilepsy

Contrary to the effectiveness of VNS in the absence seizures mimicked by the low PTZ model, acute VNS did not reduce seizure activity in genetic absence epilepsy rats from Strasbourg (GAERS) (Dedeurwaerdere, et al., 2004, Dedeurwaerdere, et al., 2005). It even prolonged the duration of the abnormal EEG pattern at the first day of stimulation.

Models for temporal lobe epilepsy

The antiseizure effect of VNS in temporal lobe epilepsy has been studied in the kindling model. Acute VNS applied 30 s prior to and 30 s after the seizure non-significantly reduced seizure duration (Naritoku, et al., 1995). In a second study, VNS was applied after the kindling stimulus an approach that completely aborted the seizures in one third of the rats (Dedeurwaerdere, et al., 2006). Application of VNS simultaneously with the kindling pulse, did not affect seizure severity in another study (Raedt, et al., 2009), while Rijkers et al (2010) demonstrated that a single three-minute pulse of VNS that was administered one minute prior to the kindling stimulus and for two minutes thereafter reduced seizure duration, while the latency to the occurrence of a generalized seizure increased in a subpopulation of the rats.

VNS can prevent and interrupt seizures

The pre-treatment effects of VNS have also been explored by applying VNS to animals prior to evoking the seizure. VNS is anticonvulsant when applied immediately before PTZ-induced seizures (Takaya, et al., 1996), seizures induced by amygdala kindling (Naritoku and Mikels, 1996), seizures evoked by cortical stimulation (De Herdt, et al., 2010), and strychnine induced seizures (Zabara, 1985, Zabara, 1992). In several studies the VNS effect outlasted the pulse itself (Lockard, et al., 1990, Zagon and Kemeny, 2000).

There is no consensus whether VNS can abort a seizure that has already started: Woodbury and Woodbury (1990) reported that such seizures can not be interrupted, while Zabara (1992) demonstrated that VNS applied after seizure initiation can interrupt or terminate the seizure, and Sunderam (2001) showed termination of a significant number of seizures during stimulation. Yang (2007) showed that the earlier applied the more effective VNS is; nevertheless, seizures that had already started could be aborted.

Chronic VNS exerts an anti-epileptic effect

Only six animal studies have been published on chronic stimulation of the vagus nerve, i.e. on stimulation applied for multiple days (for an overview see Table 8.2).
Lockard (1990) studied the effect of VNS using a chronic focal epilepsy model by injecting alumina-gel into the cortices of four rhesus monkeys after which the monkey developed spontaneous seizures. VNS was delivered at the onset of every seizure detected by a monitoring system or every three hours if VNS had not occurred in the preceding hour. Half of the animals became nearly seizure free.
VNS in experimental absence epilepsy was less successful since in GEARS intermittent application of VNS for several days did not suppress seizure activity (Dedeurwaerdere, et al., 2004, Dedeurwaerdere, et al., 2005).
Two abstracts have been published on the effect of long-term VNS in two models of temporal lobe epilepsy. Six weeks of continuous VNS reduced seizure severity in the alternate day rapid kindling model (Raedt, et al., 2009). In the status epilepticus model, twelve weeks of VNS reduced seizure frequency significantly in seven out of ten rats, with three out of ten rats showing a reduction of 50% or more (Treiman, et al., 2009).
Finally, Muñana (2002) looked at the effectiveness of VNS in dogs that suffered from naturally occurring medically refractory epilepsy by using a double-masked crossover study with 13 weeks of stimulation, 4 weeks washout and 13 weeks of no stimulation. They found a reduction in seizure frequency in four dogs, while in two dogs it remained constant and in three dogs it increased. A significant 34% seizure frequency reduction compared to baseline was found during the last four weeks of VNS.

VNS has anti-epileptogenic properties

The question if VNS exerts an effect on the development of seizures, i.e. if it has antiepileptogenic properties, is mainly a scientifically interesting question. Four studies on this subject, all in which the kindling model was used, have been published (for an overview see Table 8.2). VNS delayed the development of seizures in cats (Fernandez-Guardiola, et al., 1999), but this effect was absent when VNS was started after the cats had already reached a more severe seizure stage (Magdaleno-Madrigal, et al., 2004). Kindling in rats was slowed as well: one hour of VNS prior to the kindling pulse increased the mean number of stimuli needed to reach the generalized seizure state (Naritoku and Mikels, 1997).
These antiepileptogenic effects of VNS could not be confirmed by the most recent study, where the kindling rate did not differ between animals treated with two hours of VNS prior to the kindling stimulus and controls (Dedeurwaerdere, et al., 2006). The authors suggested that VNS treatment even could have rendered the amygdala more excitable because after discharge threshold determination evoked generalized seizures in all VNS treated animals and only in half of the controls.

Table 8.2 Chronic VNS during epileptogenesis and in epilepsy models.

	Model represents	Method of generation		Read-out parameter	VNS effect	Reference
Epilepto-genesis	Epileptogenesis	Electrical	Kindling	After discharge duration	↑	Magdaleno-Madrigal (2004)
					=	Dedeurwaerdere (2006)
				Kindling rate	↑	Fernández-Guardiola (1999), Naritoku (1997)
					=	Dedeurwaerdere (2006)
				Generalized seizure duration	↑	Dedeurwaerdere (2006)
Epilepsy	Focal epilepsy	Chemical	Aluminium hydroxide sensor motor cortex	Complete seizure abolishment	50%	Lockard (1990)
				Stabilization interseizure interval	50%	
	Temporal lobe epilepsy	Chemical	SE (lithium/ pilocarpine)	Seizure frequency	↓	Treiman (2009)
		Electrical	Kindling	Seizure severity	↓	Raedt (2009)
	Absence epilepsy	Genetic	GAERS	Number/ frequency/ duration spike wave discharge	=	Dedeurwaerdere (2004, 2005)
	Other	Spontaneous – various seizures		Decrease seizure frequency	44%	Muñana (2002)

↑ increase; ↓ decrease; =no difference.

Side effects

Different side effects of VNS have been reported. First of all cardiorespiratory function can be influenced by VNS. A decrease in heart rate and respiration as a consequence of VNS has been described in several studies (McLachlan, 1993, Osharina, et al., 2006, Roosevelt, et al., 2006, Sunderam, et al., 2001, Woodbury and Woodbury, 1990, Woodbury and Woodbury, 1991, Zabara, 1992). These cardiorespiratory effects seem to be dependent on stimulus strength (Osharina, et al., 2006, Woodbury and Woodbury, 1990, Woodbury and Woodbury, 1991, Zaaimi, et al., 2008). In another study, VNS prevented seizure-related cardiac arrhythmia, changes in blood pressure, and heart rate (Sahin, et al., 2009). A significant decrease in oxygen saturation has also been reported during VNS in rats (Sunderam, et al., 2001). However, in the most recent study VNS did not alter heart rate, cortical blood flow or mean arterial pressure, neither during baseline conditions nor during seizures (Hotta, et al., 2010).
Other side effects include increased urination and defecation, drooling, tearing (Dedeurwaerdere, et al., 2006), and behavioural responses, such as vocalization, coughing, head turning, immobilisation, licking, scratching, and swallowing (Dedeurwaerdere, et al., 2006, Dedeurwaerdere, et al., 2004, Dedeurwaerdere, et al., 2005, Fernandez-Guardiola, et al., 1999, Muñana, et al., 2002). Horner's syndrome has occurred as complication of surgical placement of the electrode (Aalbers, et al., 2009, Muñana, et al., 2002, Rijkers, et al., 2010).
Animal studies on positive side effects of VNS on cognition and mood have been performed but fall beyond the scope of this review.

Mechanism of action

All of the VNS studies described above mainly focussed on VNS effectiveness. Additionally, several studies have been published in which the mechanism of action of VNS has been explored. Based on these studies, the mechanism of action is thought to be related to the type of fibres that are recruited, desynchronization of neuronal activity, arousal, changes in neurotransmitters and hippocampal plasticity, and immunomodulation.

Which fibres are recruited by which parameters?

In the early studies of both Woodbury (1990) and Zabara (1992), seizure activity was diminished or abolished only if high stimulus intensities were used. Based on the physical properties of C-fibres, Woodbury and Zabara therefore both hypothesized that seizure suppression was the result of C-fibre activation alone. This hypothesis was rejected when another study demonstrated that selective destruction of vagal C-fibres by injection of capsaicin did not alter VNS effectiveness (Krahl, et al., 2001).

Desynchronization of neuronal activity

Based on the fact that seizures are characterized by highly synchronized EEG activity, and on the finding that VNS can alter EEG activity in animal studies (Balzamo and Jammes, 1989, Chase, et al., 1967, Chase, et al., 1966, Magnes, et al., 1961, Peñaloza-Rojas, 1964, Peñaloza-Rojas, et al., 1969), it was initially hypothesized that the main mechanism of action of VNS consisted of desynchronization of neuronal activity.
Desynchronization may be the result of VNS-induced activation of brain structures that have been shown to play a role in regulation or generation of seizures - such as the amygdala, the limbic cortex, and parts of the thalamus - and that are anatomically connected with the vagus nerve (Hopkins and Holstege, 1978, Ito and Craig, 2005, Ricardo and Koh, 1978). In line with this hypothesis, immunohistochemical studies have confirmed activation of some of these afferent sites by VNS (Cunningham, et al., 2008, Naritoku, et al., 1995, Osharina, et al., 2006). Activation of these pathways may lead to decreased cortical excitability since VNS inhibited cortical responses evoked by amygdala stimulation (Lyubashina and Panteleev, 2009) and evoked slow hyperpolarization in rat cortical neurons (Zagon and Kemeny, 2000). Involvement of the hippocampus has been indicated by VNS-induced hippocampal decreases in glucose metabolism (Dedeurwaerdere, et al., 2005) and blood flow (Hotta, et al., 2010). The importance of the NTS is underlined by two studies that demonstrated inhibition of generalized seizure development by electrical stimulation of the medial NTS in amygdala kindled cats (Magdaleno-Madrigal, et al., 2010, Magdaleno-Madrigal, et al., 2002).

Arousal

Others hypothesized that VNS exerts its anticonvulsive effect via nonspecific arousal. This hypothesis comes from several studies in rats in which aspecific types of ‘stimulation’ resulted in anticonvulsive effects as well. For instance McLachlan (1993) demonstrated that placement of rats’ tails into hot water reduced interictal spike frequency as effectively as VNS. Furthermore, trigeminal nerve stimulation reduced PTZ-induced seizures (Fanselow, et al., 2000) while electro-acupuncture inhibited cortical epileptiform activity as effectively as VNS (Zhang, et al., 2008) and sciatic nerve stimulation resulted in seizure duration reduction (Sunderam, et al., 2001). It is hypothesized that various stimuli, e.g. VNS, can activate the reticular system in the brainstem. Subsequent arousal may therefore be responsible for the effect of VNS.

Neurotransmitters

Several neurotransmitters have been implicated in the anticonvulsive mechanism of action of VNS. Norepinephrine (NE) signalling has been shown to be affected since VNS induced c-fos expression in the locus coeruleus (LC), the most important source of NE in the brain (Naritoku, et al., 1995) and increased firing of LC neurons (Dorr and

Debonnel, 2006, Groves, et al., 2005, Krahl, et al., 1994). Moreover, VNS-induced increases in NE were found in the hippocampus (Meurs, et al., 2008, Roosevelt, et al., 2006), amygdala (Hassert, et al., 2004), and cortex (Roosevelt, et al., 2006). The LC appears to be crucial for the anticonvulsive effects of VNS since seizure-suppressive effects of VNS were prevented by LC lesioning (Krahl, et al., 1994, Krahl, et al., 1998).
Serotonergic transmission may also play a role since basal firing rates of serotonergic neurons in the dorsal raphe nucleus significantly increased after chronic VNS (Dorr and Debonnel, 2006, Manta, et al., 2007, Manta, et al., 2009). This effect seems to be NE-dependent since selective lesioning of the LC prevented this enhancement of serotonin neuron firing (Manta, et al., 2009). Contradictory, VNS did not alter hippocampal serotonin levels in another study using the pilocarpine model (Meurs, et al., 2008).
The excitatory neurotransmitter glutamate is excessively released during seizures and it has been hypothesized that VNS is effective because it affects the ratio between glutamate and the inhibitory neurotransmitter gamma-amino-butyric acid (GABA). One animal study has found that animals are less susceptible to seizures when GABAergic neurotransmission is increased or glutamatergic neurotransmission decreased in the NTS (Walker, et al., 1999). However, hippocampal GABA levels were not changed after VNS (Meurs, et al., 2008).
Nitric oxide (NO) has also been implicated in the anticonvulsive mechanism of VNS since inhibition of NO synthase reversed the inhibiting effect of VNS on amgydala-evoked cortical responses (Lyubashina and Panteleev, 2009). This appears to be a local cortical effect of VNS since VNS did not activate NO-ergic neurons in the NTS in another study (Osharina, et al., 2006).

Hippocampal plasticity

Temporal lobe epilepsy is characterized by mossy fibre sprouting in the hippocampus. VNS may have the potential to affect seizure-associated changes in hippocampal plasticity: VNS increased the expression of fibroblast growth factor and brain-derived neurotrophic factor in the hippocampus and cortex of rats (Biggio, et al., 2009, Follesa, et al., 2007). Furthermore, progenitor cell proliferation in the dentate gyrus increased after 3-48 hours of VNS (Revesz, et al., 2008) and was still detectable three weeks later (Biggio, et al., 2009). Chronic VNS for four weeks on the other hand did not affect the number of proliferating cells (Biggio, et al., 2009). Survival of progenitor cells was also not enhanced by VNS (Revesz, et al., 2008).
Since both NE and serotonin can influence progenitor cell proliferation (Brezun and Daszuta, 1999, Kulkarni, et al., 2002), these VNS-induced plastic changes may be the result of reported neurotransmitter changes. Newborn neurons are less excitable (Jakubs, et al., 2006) and changes in neurogenesis may ultimately result in a less excitable network.

Immunomodulation

The vagus nerve is also implicated in immunomodulation as efferent vagus nerve fibres systemically inhibit pro-inflammatory cytokine release (Pavlov and Tracey, 2005, Pavlov, et al., 2003). Although it is still unclear to what extent VNS affects this so-called 'cholinergic anti-inflammatory pathway', VNS appears to exert an afferent neuroimmunomodulatory effect since two hours of continuous VNS induced expression of the pro-inflammatory cytokine interleukin-1beta in the hippocampus and hypothalamus of rats (Hosoi, et al., 2000).
Furthermore, VNS activates the hypothalamic-pituitary-adrenal axis. This was demonstrated by VNS-induced increased hippocampal expression of corticotrophin releasing factor and increased plasma levels of adrenocorticotropic hormone and corticosteron after VNS (De Herdt, et al., 2009, Hosoi, et al., 2000).

Discussion

VNS effectiveness

VNS has anticonvulsive effects in animal models for seizures and epilepsy. It diminishes many different partial seizures (De Herdt, et al., 2009, Godlevsky, et al., 1994, Hotta, et al., 2010, Meurs, et al., 2008, Stoica and Tudor, 1967, Stoica and Tudor, 1968, Yang, et al., 2007, Zanchetti, et al., 1952), interictal spike activity (McLachlan, 1993), tonic clonic seizures (Krahl, et al., 1998, Sahin, et al., 2009, Sunderam, et al., 2001, Woodbury and Woodbury, 1990, Woodbury and Woodbury, 1991, Zhang, et al., 2008), and temporal lobe seizures (Dedeurwaerdere, et al., 2006, Naritoku and Mikels, 1996, Raedt, et al., 2009, Rijkers, et al., 2010). Effects of VNS on absence seizures and epilepsy are varying: low dose PTZ seizures were effectively diminished (Krahl, et al., 2001, Krahl, et al., 2003, Takaya, et al., 1996), but seizure activity in GAERS was not suppressed (Dedeurwaerdere, et al., 2004, Dedeurwaerdere, et al., 2005). Pre-treatment alone can attenuate seizures and VNS effects often outlast the duration of the stimulus (De Herdt, et al., 2010, Lockard, et al., 1990, McLachlan, 1993, Naritoku and Mikels, 1996, Zabara, 1992, Zagon and Kemeny, 2000). Chronic VNS reduces seizure frequency (Lockard, et al., 1990, Muñana, et al., 2002, Treiman, et al., 2009) and severity (Raedt, et al., 2009).
However, in some of the studies VNS had proconvulsive effects (Dedeurwaerdere, et al., 2006, Meurs, et al., 2008, Stoica and Tudor, 1967, Stoica and Tudor, 1968, Sunderam, et al., 2001).

The anticonvulsive effects and effectiveness vary between the studies. The reasons for these varying results may be related the different experimental approaches.
First of all, technical aspects regarding electrode design and surgical procedure as described in Table 8.3 are an important factor. In most animal studies a detailed

description of the electrode design lacks and it is likely that differences in electrode design are present. This alone probably leads to differences in impedance, which is presumed to be high since the contact area between electrode and nerve is much smaller than in the clinical situation. It is therefore not known what stimulus intensity is actually delivered to the nerve in each of the studies. Some attempts have been made to directly measure stimulus conduction along the nerve (Woodbury and Woodbury, 1990, Woodbury and Woodbury, 1991), but this is complicated, as the stimulus artefact can disguise the stimulus itself. The experiment that directly shows that the afferent VNS pulse reaches the brain, i.e. a study in which the electrophysiological activity in the NTS is recorded directly during VNS, has not been published so far.

Importantly, in some animal studies the vagus nerve was cut to ascertain merely afferent stimulation (Hotta, et al., 2010, Stoica and Tudor, 1967, Stoica and Tudor, 1968, Zanchetti, et al., 1952). Cutting the vagus nerve affects several nuclei in the brain stem (Hopkins, 1996). It is unknown if and how these brainstem changes affect the anticonvulsive effect of VNS. Moreover, differences in manipulation of the nerve during surgical placement may influence effectiveness as well: in most studies the vagus nerve was prepared free from the carotid artery, while one study the vagus nerve electrode was wrapped around both vagus nerve and carotid artery in order to avoid nerve damage (Rijkers, et al., 2010).

Secondly, differences in experimental design have to be taken into consideration. In general, experimental group sizes are rather small. Moreover, there is considerable variation between VNS parameters used (see Table 8.3). It has been shown repeatedly that variations in stimulation parameters affect the effectiveness of VNS (McLachlan, 1993, Stoica and Tudor, 1967, Stoica and Tudor, 1968, Takaya, et al., 1996, Woodbury and Woodbury, 1990, Yang, et al., 2007, Zabara, 1992, Zagon and Kemeny, 2000, Zhang, et al., 2008, Zhang, et al., 2008) and cellular changes (Manta, et al., 2009, Osharina, et al., 2006, Zagon and Kemeny, 2000, Zhang, et al., 2008). This makes it difficult to interpret and compare results from different studies.

Some studies have been performed under general anaesthesia or in immobilized animals (Godlevsky, et al., 1994, Hotta, et al., 2010, McLachlan, 1993, Stoica and Tudor, 1967, Stoica and Tudor, 1968, Sunderam, et al., 2001, Woodbury and Woodbury, 1990, Yang, et al., 2007, Zabara, 1985, Zabara, 1992, Zagon and Kemeny, 2000, Zanchetti, et al., 1952, Zhang, et al., 2008), while others have been performed in awake and freely moving animals (De Herdt, et al., 2010, Dedeurwaerdere, et al., 2006, Dedeurwaerdere, et al., 2004, Dedeurwaerdere, et al., 2005, Fernandez-Guardiola, et al., 1999, Krahl, et al., 2003, Lockard, et al., 1990, Magdaleno-Madrigal, et al., 2004, Meurs, et al., 2008, Muñana, et al., 2002, Naritoku and Mikels, 1996, Naritoku and Mikels, 1997, Raedt, et al., 2009, Rijkers, et al., 2010, Sahin, et al., 2009, Takaya, et al., 1996, Treiman, et al., 2009, Woodbury and Woodbury, 1990, Woodbury and Woodbury, 1991) (see also Table 8.3).

Table 8.3 Technical aspects of VNS studies in animal models of seizures and epilepsy.

	# Animals (total)	# Animals (/group)	Awake / Anaesthetized	Anaesthetic	Electrode	Nerve section	Acute/ Chronic	Timing	Freq (Hz)	Current (mA)	Pulse width (μs)	On (min)	Off (min)	Duration (min)	Outcome measure
Dedeurwaerdere 2004	21	2-8	Awake	n.a.	Cuff	n.m.	Acute	D	30	1.5±0.75	500	n.a.	n.a.	60	EEG
							Chronic 5d	C	30	1.5	500	0.5	5	180	EEG
Dedeurwaerdere 2005	39	8-18	Awake	n.a.	Cuff	n.m.	Acute	D	30	1.5	500	n.a.	n.a.	60	EEG
							Chronic 7d	C	30	1.5	500	1	0.2	C	EEG
Dedeurwaerdere 2006	16	6-10	Awake	n.a.	Cuff	n.m.	Acute	A	30	0.25-0.5	500	0.5	1.1	1	EEG/ behaviour
							Chronic with kindling	P	30	0.25-0.5	500	0.5	1.1	120	EEG/ behaviour
De Herdt 2010	8	8	Awake	n.a.	Cuff	n.m.	Acute	P	30	0.74	250	0.5	1.8	60	Behaviour
Fernández-Guardiola 1999	15	5-10	Awake	n.a.	Hook	n.m.	Chronic with kindling	PDA	30	1.2-2.0	500	n.a.	n.a.	1	Behaviour
Godlevsky 1994	n.m.	n.m.	Anaesthetized	n.m.	n.m.	n.m.	Acute	n.m.	100-300	n.m.	n.m.	n.m.	n.m.	n.m.	EEG
Hotta 2010	13	2-6	Anaesthetized	Urethane	Linear	Yes	Acute	D	2-50	n.m.	500	n.a.	n.a.	0.3-1	EEG
Krahl 1998	52	10-15	Awake	n.m.	Cuff	n.m.	Acute	PD	20	0.8	500	n.a.	n.a.	0.5+test duration	Behaviour
Krahl 2001	34	7-10	Awake	n.m.	Cuff	n.m.	Acute	PD	20	1	500	n.a.	n.a.	15.5	Behaviour
Krahl 2003	16	7-9	Awake	n.a.	Cuff	n.m.	Acute	PD	20	1	500	n.a.	n.a.	0.5	Behaviour
Lockard 1990	4	4	Awake	n.a.	Cuff	n.m.	Chronic	every 3h/ D	50-250	3/5/7	500/600	n.a.	n.a.	SD/0.6	EEG/ behaviour
Magdaleno-Madrigal 2004	3	3	Awake	n.a.	Hook	No	Chronic with kindling	n.m.	n.m.	n.m.	n.m.	1	5	60	Behaviour
McLachlan 1993	15	5	Anaesthetized	Urethane	n.m.	n.m.	Acute	A	20/50	0.01-1.2	500	n.a.	n.a.	1/60-1/3	ECoG
	4	1-4	Anaesthetized	Urethane	n.m.	n.m.	Acute	D	n.m.	1/1.2	n.m.	n.a.	n.a.	SD	EcoG
Meurs 2008	n.m.	n.m.	Awake	n.a.	n.m.	n.m.	Acute	PD	n.m.	n.m.	n.m.	n.m.	n.m.	SD+120	Behaviour
Muñana et al 2002	10	10	Awake	n.a.	Spiral	n.m.	Chronic 13w	C	30	Adj.	550	0.5	5	C	Behaviour
Naritoku et al 1996	7	7	Awake	n.a.	Cuff	n.m	Acute	P/ PDA	30	1	500	n.a.	n.a.	1/60	EEG/ behaviour

	# Animals (total)	# Animals (/group)	Awake / Anaesthetized	Anaesthetic	Electrode	Nerve section	Acute/ Chronic	Timing	Freq (Hz)	Current (mA)	Pulse width (µs)	On (min)	Off (min)	Duration (min)	Outcome measure
Naritoku et al 1997	8	4	Awake	n.a.	n.m.	n.m.	Chronic with kindling	P	30	1	500	n.a.	n.a.	60	Behaviour
Raedt et al 2009	19	6-13	Awake	n.a.	Cuff	n.m.	Acute	D	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	EEG/ behaviour
							Chronic 6w	C	n.m.	n.m.	n.m.	n.m.	n.m.	C	EEG/ behaviour
Rijkers et al 2009	31	8-12	Awake	n.a.	Circular	n.m.	Acute	PDA	30	1	500	0.5	0.2	3	EEG/ behaviour
Sahin et al 2009	21	7	Awake	n.a.	Cuff	n.m.	Acute	PD	20	1	500	n.a.	n.a.	SD +10	EEG/ behaviour
Stoica et al 1967	12	12	Awake (immobilized)	n.a.	n.m.	Yes	Acute	A	20-25	n.m.	300	n.a.	n.a.	n.m.	ECoG
Stoica et al 1968	11	11	Awake (immobilized)	n.a.	n.m.	Yes	Acute	A	20-25	n.m.	300	n.a.	n.a.	n.m.	ECoG
Sunderam et al 2001	44	11	Anaesthetized	Ketamine-Acepromazine	n.m.	n.m.	Acute	A	20	1.4	500	0.5	2	130	ECoG
Takaya et al 1996	29	6-12	Awake	n.a.	Cuff	n.m.	Acute	P	30	1	500	0.5	5	60/1	EEG/ behaviour
Treiman et al 2009	n.m.	10	Awake	n.a.	n.m.	n.m.	Chronic 12w	C	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	EEG
Woodbury et al 1990	n.m.	n.m.	Anaesthetized	Ketamine-Acepromazine	Cuff/ hook	n.m.	Acute	D	1-20	200-800	500	n.a.	n.a.	n.m./1	ECoG/ EMG
	n.m.	n.m.	Awake	n.a.	Cuff	n.m.	Acute	PDA	10/20	n.m.	500	n.a.	n.a.	1	EMG/ behaviour
Woodbury et al 1991	n.m.	15	Awake	n.a.	Cuff	n.m.	Acute	PDA	10-30	Adj.	500	n.a.	n.a.	0.3-0.5	EEG/ EMG/ behaviour
Yang et al 2007	20	8-12	Anaesthetized	Ketamine	n.m.	n.m.	Acute	PD	20	n.m.	120	n.a.	n.a.	0.1-0.6	EMG

	# Animals (total)	# Animals (/group)	Awake / Anaesthetized	Anaesthetic	Electrode	Nerve section	Acute/ Chronic	Timing	Freq (Hz)	Current (mA)	Pulse width (µs)	On (min)	Off (min)	Duration (min)	Outcome measure
Zabara 1985	n.m.	n.m.	Anaesthetized	n.m.	Cuff	n.m.	Acute	PD	20-150	1-10	100-1000	n.a.	n.a.	n.m.	EMG
Zabara 1992	20	n.m.	Anaesthetized	A-chloralose	Cuff/ hook	n.m.	Acute	A	20-150	3-100	200-2000	n.a.	n.a.	n.m.	EMG
Zanchetti et al 1956	15	15	Anaesthetized	Ether	n.m.	Yes	Acute	A	2-300	n.m.	500	n.a.	n.a.	n.m.	EEG
Zhang et al 2008	20	20	Anaesthetized	Xylazine/ ketamine	Hook	n.m.	Acute	A	30	1/3	500	n.a.	n.a.	5	Field potential

#number; freq=frequency; Hz=Herz; mA=milli ampere; µs=micro seconds; min=minutes; n.a.=not applicable; n.m.=not mentioned; d=days; w=weeks; P=prior to seizure; D=during seizure; A=after seizure; C=continuous; SD=stimulus duration; ECoG=electrocorticogram; EEG=electroencephalogram; EMG=electromyogram; adj.adjusted

This hampers the interpretation of the results since it has been shown that anaesthesia influences VNS: unanaesthetized animals need higher currents than anaesthetized animals for obtaining an anticonvulsive effect (Woodbury and Woodbury, 1990). Woodbury and Woodbury (1990) attributed this need for higher VNS currents to an increase of shunting of body fluids in freely moving animals favouring current spread. However, the anaesthetic used, ketamine, has anticonvulsive properties of its own (Modica, et al., 1990). Secondly, dampening of neuronal activity by anaesthesia in general may have contributed to the lower VNS intensity needed during anaesthesia. After all, induction of arousal may be one of the mechanisms of action of VNS and arousal is diminished during general anaesthesia. It is therefore possible that by interfering with the state of arousal, VNS effectiveness is affected too.

Moreover, many different seizure and epilepsy models have been used (Table 8.1). On the one hand the effects of VNS on all these pathophysiologically different seizures demonstrate the broad antiseizure potency of VNS. On the other hand, the studies that evaluated the anticonvulsant effect in seizures evoked in non-epileptic animals (upper part Table 8.1), may have overrated VNS efficacy, because seizures induced in epileptic and non-epileptic animals respond differently to various treatments (Loscher, 1997). Therefore, results from studies that used chronic epilepsy models, such as the status epilepticus model (Treiman, et al., 2009) or the kindling model (Dedeurwaerdere, et al., 2006, Naritoku and Mikels, 1996, Raedt, et al., 2009, Rijkers, et al., 2010), are most interesting. Several studies have used the kindling model to evaluate the antiepileptogenic properties of VNS by treating the animals with VNS during kindling acquisition (Dedeurwaerdere, et al., 2006, Fernandez-Guardiola, et al., 1999, Magdaleno-Madrigal, et al., 2004, Naritoku and Mikels, 1997). The validity of these results can be debated, since it is still unclear whether the increase in kindling rate results from a repeated antiseizure effect of VNS (repeatedly preventing or diminishing seizure occurrence during the kindling process) or whether it reflects a true antiepileptogenic effect. The option that a repeated antiseizure effect, and not an antiepileptogenic effect is obtained, is supported by the absence of any anti-epiletogenic effects if VNS was initiated after animals already experienced more severe (Magdaleno-Madrigal, et al., 2004).

Finally, comparison of VNS effects is hampered by the difference in outcome measures (Table 8.3): in some studies only behavioural parameters were assessed (Krahl, et al., 2003, Meurs, et al., 2008, Muñana, et al., 2002, Naritoku and Mikels, 1997), while other studies concentrated on electrophysiological parameters such as EEG (Dedeurwaerdere, et al., 2004, Dedeurwaerdere, et al., 2005, Hotta, et al., 2010, McLachlan, 1993, Treiman, et al., 2009, Woodbury and Woodbury, 1991), electrocorticogram (Stoica and Tudor, 1967, Stoica and Tudor, 1968, Sunderam, et al., 2001), and electromyogram (Woodbury and Woodbury, 1990, Woodbury and Woodbury, 1991, Yang, et al., 2007, Zabara, 1985, Zabara, 1992, Zhang, et al., 2008). Several studies combined behavioural analysis and electrophysiological recordings (De Herdt, et al., 2010, Dedeurwaerdere, et al., 2006, Fernandez-Guardiola, et al., 1999,

Lockard, et al., 1990, Magdaleno-Madrigal, et al., 2004, Raedt, et al., 2009, Rijkers, et al., 2010, Sahin, et al., 2009, Takaya, et al., 1996).

Clinical relevance

In addition to the large variation in experimental design and technical details among the different studies, several other limitations prevent the extrapolation of animal data to humans.

First of all, in the vast majority of the experiments VNS was acutely applied in close temporal proximity to the seizure. This only mimics the clinical situation of activation of the pulse generator with the hand-held magnet when perceiving an upcoming seizure. This type of stimulation may be important for some patients but is not responsible for the treatment effect in the majority of patients that benefit from VNS.

Moreover, in most of the animal studies VNS consists of a single train of pulses while patients receive chronic cyclic stimulation for years. This difference is important, because the results of VNS appear to improve as the period of treatment lengthens (DeGiorgio, et al., 2000), reaching a plateau after one to two years (Morris and Mueller, 1999).

Finally, VNS is indicated in patients with pharmacotherapy resistant epilepsy. Only one animal study has evaluated the effect of VNS in refractory animals (Muñana, et al., 2002). As the existence of responders and non-responders is a well-known phenomenon in pharmacological animal studies on the effectiveness of antiepileptic drugs (Brandt, et al., 2004, Cramer, et al., 1998, Ebert and Loscher, 1999, Ebert, et al., 1999), VNS efficacy might have been over or underestimated by not preselecting pharmacoresistant animals.

These limitations call for more extensive research. To maximize the clinical relevance and interpretation of data we recommend the following:

1) VNS treatment should be chronic, i.e. for multiple days, for example by implanting animals with a small pulse generator.
2) VNS parameters should be comparable to clinically used parameters.
3) When receiving VNS, animals should be unaenesthetized and freely moving.
4) Antiepileptic effectiveness of VNS should be evaluated in chronic epilepsy models.
5) Pharmaco-responsive status of the animals should be taken into account.

These future studies should be directed at further elucidation of the mechanism of action. More knowledge about the pathways and networks involved may eventually lead to a more effective target for antiepileptic neuromodulation. Furthermore, these future studies should make an attempt to identify VNS responder characteristics in order to find (bio)markers that can be used in clinical practice to identify those patients that are most likely to respond to the treatment.

Conclusion

Thirty-three experimental studies have been conducted in which VNS was investigated as an anticonvulsant treatment. These studies have demonstrated the safety and effectiveness of VNS, but the mechanism of action has not been fully elucidated. In light of the growing interest in, and the broader application of VNS, further research is warranted. Future studies should apply chronic VNS in a chronic animal model for epilepsy.

References

Aalbers, M. W., Rijkers, K., van Winden, L. A., Hoogland, G., Vles, J. S., and Majoie, H. J., 2009. Horner's syndrome: A complication of experimental carotid artery surgery in rats. Auton Neurosci 147**,** 64-69.

Aghajanian, G. K., and Wang, R. Y., 1977. Habenular and other midbrain raphe afferents demonstrated by a modified retrograde tracing technique. Brain Res 122**,** 229-242.

Asala, S. A., and Bower, A. J., 1986. An electron microscope study of vagus nerve composition in the ferret. Anat Embryol (Berl) 175**,** 247-253.

Bailey, P., and Bremer, 1938. A sensory cortical representation of the vagus nerve: with a note on the effects of low blood pressure on the cortical electrogram. J Neurophysiol 1**,** 405-412.

Balzamo, E., and Jammes, Y., 1989. Vagal afferents and EEG rhythms in the Sl area in anesthetized cats: similarities between responses to electrical and chemical (phenyldiguanide) stimulations. Arch Int Physiol Biochim 97**,** 483-492.

Beekwilder, J. P., and Beems, T., 2010. Overview of the clinical applications of vagus nerve stimulation. J Clin Neurophysiol 27**,** 130-138.

Biggio, F., Gorini, G., Utzeri, C., Olla, P., Marrosu, F., Mocchetti, I., and Follesa, P., 2009. Chronic vagus nerve stimulation induces neuronal plasticity in the rat hippocampus. Int J Neuropsychopharmacol 12**,** 1209-1221.

Brezun, J. M., and Daszuta, A., 1999. Depletion in serotonin decreases neurogenesis in the dentate gyrus and the subventricular zone of adult rats. Neuroscience 89**,** 999-1002.

Castle, M., Comoli, E., and Loewy, A. D., 2005. Autonomic brainstem nuclei are linked to the hippocampus. Neuroscience 134**,** 657-669.

Chase, M. H., Nakamura, Y., Clemente, C. D., and Sterman, M. B., 1967. Afferent vagal stimulation: neurographic correlates of induced EEG synchronization and desynchronization. Brain Res 5**,** 236-249.

Chase, M. H., Sterman, M. B., and Clemente, C. D., 1966. Cortical and subcortical patterns of response to afferent vagal stimulation. Exp Neurol 16**,** 36-49.

Cunningham, J. T., Mifflin, S. W., Gould, G. G., and Frazer, A., 2008. Induction of c-Fos and DeltaFosB immunoreactivity in rat brain by Vagal nerve stimulation. Neuropsychopharmacology 33**,** 1884-1895.

De Herdt, V., De Waele, J., Raedt, R., Wyckhuys, T., El Tahry, R., Vonck, K., Wadman, W., and Boon, P., 2010. Modulation of seizure threshold by vagus nerve stimulation in an animal model for motor seizures. Acta Neurol Scand 121**,** 271-276.

De Herdt, V., Puimege, L., De Waele, J., Raedt, R., Wyckhuys, T., El Tahry, R., Libert, C., Wadman, W., Boon, P., and Vonck, K., 2009. Increased rat serum corticosterone suggests immunomodulation by stimulation of the vagal nerve. J Neuroimmunol 212**,** 102-105.

Dedeurwaerdere, S., Cornelissen, B., Van Laere, K., Vonck, K., Achten, E., Slegers, G., and Boon, P., 2005. Small animal positron emission tomography during vagus nerve stimulation in rats: a pilot study. Epilepsy Res 67**,** 133-141.

Dedeurwaerdere, S., Gilby, K., Vonck, K., Delbeke, J., Boon, P., and McIntyre, D., 2006. Vagus nerve stimulation does not affect spatial memory in fast rats, but has both anti-convulsive and pro-convulsive effects on amygdala-kindled seizures. Neuroscience 140**,** 1443-1451.

Dedeurwaerdere, S., Vonck, K., Claeys, P., Van Hese, P., D'Have, M., Grisar, T., Naritoku, D., and Boon, P., 2004. Acute vagus nerve stimulation does not suppress spike and wave discharges in genetic absence epilepsy rats from Strasbourg. Epilepsy Res 59**,** 191-198.

Dedeurwaerdere, S., Vonck, K., Van Hese, P., Wadman, W., and Boon, P., 2005. The acute and chronic effect of vagus nerve stimulation in genetic absence epilepsy rats from Strasbourg (GAERS). Epilepsia 46 Suppl 5**,** 94-97.

DeGiorgio, C. M., Schachter, S. C., Handforth, A., Salinsky, M., Thompson, J., Uthman, B., Reed, R., Collins, S., Tecoma, E., Morris, G. L., Vaughn, B., Naritoku, D. K., Henry, T., Labar, D., Gilmartin, R., Labiner, D., Osorio, I., Ristanovic, R., Jones, J., Murphy, J., Ney, G., Wheless, J., Lewis, P., and Heck, C., 2000. Prospective long-term study of vagus nerve stimulation for the treatment of refractory seizures. Epilepsia 41**,** 1195-1200.

Dell, P., and Olson, R., 1951. [Secondary mesencephalic, diencephalic and amygdalian projections of vagal visceral afferences.]. C R Seances Soc Biol Fil 145**,** 1088-1091.

Dell, P., and Olson, R., 1951. [Thalamic, cortical and cerebellar projections of vagal visceral afferences.]. C R Seances Soc Biol Fil 145**,** 1084-1088.

Dorr, A. E., and Debonnel, G., 2006. Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission. J Pharmacol Exp Ther 318**,** 890-898.

Fanselow, E. E., Reid, A. P., and Nicolelis, M. A., 2000. Reduction of pentylenetetrazole-induced seizure activity in awake rats by seizure-triggered trigeminal nerve stimulation. J Neurosci 20**,** 8160-8168.

Fernandez-Guardiola, A., Martinez, A., Valdes-Cruz, A., Magdaleno-Madrigal, V. M., Martinez, D., and Fernandez-Mas, R., 1999. Vagus nerve prolonged stimulation in cats: effects on epileptogenesis (amygdala electrical kindling): behavioral and electrographic changes. Epilepsia 40**,** 822-829.

Follesa, P., Biggio, F., Gorini, G., Caria, S., Talani, G., Dazzi, L., Puligheddu, M., Marrosu, F., and Biggio, G., 2007. Vagus nerve stimulation increases norepinephrine concentration and the gene expression of BDNF and bFGF in the rat brain. Brain Res 1179**,** 28-34.

Fulwiler, C. E., and Saper, C. B., 1984. Subnuclear organization of the efferent connections of the parabrachial nucleus in the rat. Brain Res 319**,** 229-259.

Godlevsky, L., Shandra, A., and Mazarati, A., 1994. Effect of Vagus Stimulation on Epileptic Activity in Rats. Epilepsia 35**,** 39.

Granata, A. R., and Kitai, S. T., 1989. Intracellular study of nucleus parabrachialis and nucleus tractus solitarii interconnections. Brain Res 492**,** 281-292.

Groves, D. A., Bowman, E. M., and Brown, V. J., 2005. Recordings from the rat locus coeruleus during acute vagal nerve stimulation in the anaesthetised rat. Neurosci Lett 379**,** 174-179.

Hassert, D. L., Miyashita, T., and Williams, C. L., 2004. The effects of peripheral vagal nerve stimulation at a memory-modulating intensity on norepinephrine output in the basolateral amygdala. Behav Neurosci 118**,** 79-88.

Hopkins, D. A., Biegel, D., de Vente, J., Steinbusch, H.W.M., 1996. Vagal efferent projections: viscerotopy, neurochemistry and effects of vagotomy. Progress in Brain Research 107**,** 18.

Hopkins, D. A., and Holstege, G., 1978. Amygdaloid projections to the mesencephalon, pons and medulla oblongata in the cat. Exp Brain Res 32**,** 529-547.

Hosoi, T., Okuma, Y., and Nomura, Y., 2000. Electrical stimulation of afferent vagus nerve induces IL-1beta expression in the brain and activates HPA axis. Am J Physiol Regul Integr Comp Physiol 279**,** R141-147.

Hotta, H., Watanabe, N., Orman, R., and Stewart, M., 2010. Efferent and afferent vagal actions on cortical blood flow and kainic acid-induced seizure activity in urethane anesthetized rats. Auton Neurosci 156**,** 144-148.

Ito, S., and Craig, A. D., 2005. Vagal-evoked activity in the parafascicular nucleus of the primate thalamus. J Neurophysiol 94**,** 2976-2982.

Jakubs, K., Nanobashvili, A., Bonde, S., Ekdahl, C. T., Kokaia, Z., Kokaia, M., and Lindvall, O., 2006. Environment matters: synaptic properties of neurons born in the epileptic adult brain develop to reduce excitability. Neuron 52**,** 1047-1059.

Juhasz, G., Detari, L., and Kukorelli, T., 1985. Effects of hypnogenic vagal stimulation on thalamic neuronal activity in cats. Brain Res Bull 15**,** 437-441.

Krahl, S. E., Browning, R. A., Clark, K. B., and Smith, D. C., 1994. Possible mechanism of the Seizure Attenuating Effects of Vagus Nerve Stimulation. Society for Neuroscience Abstracts 20**,** 1453.

Krahl, S. E., Clark, K. B., Smith, D. C., and Browning, R. A., 1998. Locus coeruleus lesions suppress the seizure-attenuating effects of vagus nerve stimulation. Epilepsia 39**,** 709-714.

Krahl, S. E., Senanayake, S. S., and Handforth, A., 2001. Destruction of peripheral C-fibers does not alter subsequent vagus nerve stimulation-induced seizure suppression in rats. Epilepsia 42**,** 586-589.

Krahl, S. E., Senanayake, S. S., and Handforth, A., 2003. Right-sided vagus nerve stimulation reduces generalized seizure severity in rats as effectively as left-sided. Epilepsy Res 56**,** 1-4.

Kulkarni, V. A., Jha, S., and Vaidya, V. A., 2002. Depletion of norepinephrine decreases the proliferation, but does not influence the survival and differentiation, of granule cell progenitors in the adult rat hippocampus. Eur J Neurosci 16**,** 2008-2012.

Lanska, D. J., 2002. J.L. Corning and vagal nerve stimulation for seizures in the 1880s. Neurology 58**,** 452-459.

Lockard, J. S., Congdon, W. C., and DuCharme, L. L., 1990. Feasibility and safety of vagal stimulation in monkey model. Epilepsia 31 Suppl 2**,** S20-26.

Loscher, W., 1997. Animal models of intractable epilepsy. Prog Neurobiol 53**,** 239-258.

Lyubashina, O., and Panteleev, S., 2009. Effects of cervical vagus nerve stimulation on amygdala-evoked responses of the medial prefrontal cortex neurons in rat. Neurosci Res 65**,** 122-125.

MacLean, P. D., 1990. The triune brain in evolution : role in paleocerebral functions. Plenum press, New York, pp. 468.

Magdaleno-Madrigal, V., Valdes-Cruz, A., Martinez-Vargas, D., Almazan-Alvarado, S., Fernandez-Mas, R., and Fernandez-Guardiola, A., 2004. Effect of vagus nerve stimulation on later stages of amygdaloid kindling in freely-moving cats. FENS abstr. 2**,** A124.118

Magdaleno-Madrigal, V. M., Martinez-Vargas, D., Valdes-Cruz, A., Almazan-Alvarado, S., and Fernandez-Mas, R., 2010. Preemptive effect of nucleus of the solitary tract stimulation on amygdaloid kindling in freely moving cats. Epilepsia 51**,** 438-444.

Magdaleno-Madrigal, V. M., Valdes-Cruz, A., Martinez-Vargas, D., Martinez, A., Almazan, S., Fernandez-Mas, R., and Fernandez-Guardiola, A., 2002. Effect of electrical stimulation of the nucleus of the solitary tract on the development of electrical amygdaloid kindling in the cat. Epilepsia 43**,** 964-969.

Magnes, J., Moruzzi, G., and Pompeiano, O., 1961. Synchronization of the EEG produced by low frequency electrical stimulation of the region of the solitary tract. Arch Ital Biol 99**,** 33-67.

Manta, S., Dong, J., Debonnel, G., and Blier, P., 2007. Vagus nerve stimulation: effects on noradrenergic neuronal firing and serotonin transmission in the rat brain. Eur. Neuropsychopharmacol. 17**,** S368-S369.

Manta, S., Dong, J., Debonnel, G., and Blier, P., 2009. Enhancement of the function of rat serotonin and norepinephrine neurons by sustained vagus nerve stimulation. J Psychiatry Neurosci 34**,** 272-280.

Manta, S., Dong, J., Debonnel, G., and Blier, P., 2009. Optimization of vagus nerve stimulation parameters using the firing activity of serotonin neurons in the rat dorsal raphe. Eur Neuropsychopharmacol 19**,** 250-255.

McLachlan, R. S., 1993. Suppression of interictal spikes and seizures by stimulation of the vagus nerve. Epilepsia 34, 918-923.

Meurs, A., Clinckers, R., Raedt, R., El Tahry, R., De Herdt, V., Vonck, K., Smolders, I., Michotte, Y., and Boon, P., 2008. Vagus nerve stimulation suppressess pilocarpine-induced limbic seizures and increases hippocampal extracellular noradrenalin concentration. Epilepsia 49, 350.

Modica, P. A., Tempelhoff, R., and White, P. F., 1990. Pro- and anticonvulsant effects of anesthetics (Part II). Anesth Analg 70, 433-444.

Muñana, K. R., Vitek, S. M., Tarver, W. B., Saito, M., Skeen, T. M., Sharp, N. J., Olby, N. J., and Haglund, M. M., 2002. Use of vagal nerve stimulation as a treatment for refractory epilepsy in dogs. J Am Vet Med Assoc 221, 977-983.

Naritoku, D. K., and Mikels, J. A., 1996. Vagus nerve stimulation (VNS) attenuates electrically kindled seizures. Epilepsia 37, 75.

Naritoku, D. K., and Mikels, J. A., 1997. Vagus Nerve Stimulation (VNS) is Antiepileptogenic in the Electrical Kindling Model. Epilepsia 38, 220.

Naritoku, D. K., Terry, W. J., and Helfert, R. H., 1995. Regional induction of fos immunoreactivity in the brain by anticonvulsant stimulation of the vagus nerve. Epilepsy Res 22, 53-62.

O'Brien, J. H., Pimpaneau, A., and Albe-Fessard, D., 1971. Evoked cortical responses to vagal, laryngeal and facial afferents in monkeys under chloralose anaesthesia. Electroencephalogr Clin Neurophysiol 31, 7-20.

Osharina, V., Bagaev, V., Wallois, F., and Larnicol, N., 2006. Autonomic response and Fos expression in the NTS following intermittent vagal stimulation: importance of pulse frequency. Auton Neurosci 126-127, 72-80.

Paintal, A. S., 1973. Vagal sensory receptors and their reflex effects. Physiol Rev 53, 159-227.

Pavlov, V. A., and Tracey, K. J., 2005. The cholinergic anti-inflammatory pathway. Brain Behav Immun 19, 493-499.

Pavlov, V. A., Wang, H., Czura, C. J., Friedman, S. G., and Tracey, K. J., 2003. The cholinergic anti-inflammatory pathway: a missing link in neuroimmunomodulation. Mol Med 9, 125-134.

Peñaloza-Rojas, J. H., 1964. Electroencephalographic Synchronization Resulting from Direct Current Application to the Vagus Nerves. Exp Neurol 9, 367-371.

Peñaloza-Rojas, J. H., Barrera-Mera, B., and Kubli-Garfias, C., 1969. Behavioral and brain electrical changes after vagal stimulation. Exp Neurol 23, 378-383.

Penry, J. K., and Dean, J. C., 1990. Prevention of intractable partial seizures by intermittent vagal stimulation in humans: preliminary results. Epilepsia 31 Suppl 2, S40-43.

Pitkanen, A., Schwarzkoin, P. A., and Moshe, S. L., 2006. Models of Seizures and Epilepsy. Elsevier Academic press, Amsterdam.

Raedt, R., Waterschoot, L., Wyckhuys, T., De Herdt, V., El Tahry, R., Delbeke, J., Vonck, K., Wadman, W., and Boon, P., 2009. Effect of acute and long-term vagus nerve stimulation in fully kindled rats. Epilepsia 50, 93.

Revesz, D., Tjernstrom, M., Ben-Menachem, E., and Thorlin, T., 2008. Effects of vagus nerve stimulation on rat hippocampal progenitor proliferation. Exp Neurol 214, 259-265.

Ricardo, J. A., and Koh, E. T., 1978. Anatomical evidence of direct projections from the nucleus of the solitary tract to the hypothalamus, amygdala, and other forebrain structures in the rat. Brain Res 153, 1-26.

Rijkers, K., Aalbers, M., Hoogland, G., van Winden, L., Vles, J., Steinbusch, H., and Majoie, M., 2010. Acute seizure-suppressing effect of vagus nerve stimulation in the amygdala kindled rat. Brain Res 1319, 155-163.

Roosevelt, R. W., Smith, D. C., Clough, R. W., Jensen, R. A., and Browning, R. A., 2006. Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat. Brain Res 1119, 124-132.

Sahin, D., Ilbay, G., Imal, M., Bozdogan, O., and Ates, N., 2009. Vagus nerve stimulation suppresses generalized seizure activity and seizure-triggered postictal cardiac rhythm changes in rats. Physiol Res 58, 345-350.

Stoica, I., and Tudor, I., 1967. Effects of vagus efferents on strychninic focus of coronal gyrus. Rev Roum Neurolog 4, 287-295.

Stoica, I., and Tudor, I., 1968. Vagal trunk stimulation influences on epileptic spiking focus activity. Rev Roum Neurolog 5, 203-210.

Sunderam, S., Osorio, I., Watkins, J. F., 3rd, Wilkinson, S. B., Frei, M. G., and Davis, R. E., 2001. Vagal and sciatic nerve stimulation have complex, time-dependent effects on chemically-induced seizures: a controlled study. Brain Res 918, 60-66.

Takaya, M., Terry, W. J., and Naritoku, D. K., 1996. Vagus nerve stimulation induces a sustained anticonvulsant effect. Epilepsia 37, 1111-1116.

Treiman, L. J., Marsh, S., Sabesan, S., Ferguson, R., and Treiman, D. M., 2009. VNS-induced reduction of seizures and changes in gene expression in a rat model of chronic epilepsy. Epilepsia 50, 388.

Van Bockstaele, E. J., Peoples, J., and Telegan, P., 1999. Efferent projections of the nucleus of the solitary tract to peri-locus coeruleus dendrites in rat brain: evidence for a monosynaptic pathway. J Comp Neurol 412, 410-428.

Walker, B. R., Easton, A., and Gale, K., 1999. Regulation of limbic motor seizures by GABA and glutamate transmission in nucleus tractus solitarius. Epilepsia 40, 1051-1057.

Woodbury, D. M., and Woodbury, J. W., 1990. Effects of vagal stimulation on experimentally induced seizures in rats. Epilepsia 31 Suppl 2, S7-19.

Woodbury, J. W., and Woodbury, D. M., 1991. Vagal stimulation reduces the severity of maximal electroshock seizures in intact rats: use of a cuff electrode for stimulating and recording. Pacing Clin Electrophysiol 14, 94-107.

Yang, H., Peng, K., Hu, S., and Liu, Y., 2007. Inhibiting effect of vagal nerve stimulation to seizures in epileptic process of rats. Neuroscience Bulletin 23, 336-340.

Zaaimi, B., Grebe, R., and Wallois, F., 2008. Animal model of the short-term cardiorespiratory effects of intermittent vagus nerve stimulation. Auton Neurosci 143, 20-26.

Zabara, J., 1985. Peripheral control of hypersynchronous discharge in epilepsy. Electroencephalography and clinical neurophysiology 61, S162.

Zabara, J., 1992. Inhibition of experimental seizures in canines by repetitive vagal stimulation. Epilepsia 33, 1005-1012.

Zagon, A., and Kemeny, A. A., 2000. Slow hyperpolarization in cortical neurons: a possible mechanism behind vagus nerve simulation therapy for refractory epilepsy? Epilepsia 41, 1382-1389.

Zanchetti, A., Wang, S. C., and Moruzzi, G., 1952. [Effect of afferent vagal stimulation on the electroencephalogram of the cat in cerebral isolation.]. Boll Soc Ital Biol Sper 28, 627-628.

Zhang, J. L., Zhang, S. P., and Zhang, H. Q., 2008. Antiepileptic effect of electroacupuncture vs. vagus nerve stimulation in the rat thalamus. Neurosci Lett 441, 183-187.

Zhang, J. L., Zhang, S. P., and Zhang, H. Q., 2008. Antiepileptic effects of electroacupuncture vs vagus nerve stimulation on cortical epileptiform activities. J Neurol Sci 270, 114-121.

Chapter 9

General discussion

Inflammation

Epilepsy is one of the most common neurological disorders. Approximately one third of all patients are resistant to current drug therapies (Kwan and Brodie, 2000, Sander, 1993). Moreover, the available drugs are mainly symptomatic and do not influence disease development or disease progression. Therefore, the development of novel antiepileptic treatments is needed. This development could be aided by elucidation of the pathophysiological mechanisms underlying epilepsy. Over the last years inflammation has been implicated as one of these possible pathophysiological mechanisms (Vezzani, et al., 2011). The clinical studies that evaluated cytokine changes in epilepsies without a prototypical inflammatory aetiology were reviewed in chapter 2 of this thesis. These studies found increased levels of interleukin (IL)-6 regardless of the tissue that was analyzed. IL-1β levels were only increased in brain specimens, whereas analyses of tumor necrosis factor alpha were inconclusive. It is unclear whether these cytokine changes are intrinsic to the epilepsy itself or whether they are related to the underlying pathology. For instance, markers of inflammation were only present in hippocampal specimens from patients with hippocampal sclerosis (HS), but not in hippocampal specimens of patients without HS (Crespel, et al., 2002, Ravizza, et al., 2008). Therefore, we evaluated whether HS influences cytokine expression by comparing a broad array of cytokines in TLE patients with and without HS (chapter 3). Levels of IL-1α and IL-1 receptor antagonist were lower in sclerotic hippocampi compared to histologically normal cortex samples from the same patients. Remarkably, we were not able to demonstrate higher levels of pro-inflammatory cytokines in HS. This suggests that the presence of HS does not determine pro-inflammatory cytokine expression. A drawback of our design was that we could not directly answer the question whether the cytokine levels in our epilepsy patients are different from basal cytokine expression, because we did not compare cytokine levels to a normal non-neurological control group. Previous studies reported similar cytokine levels in brain tissue of autopsy controls and in normal brain tissue of epilepsy patients that are similar to our controls (Ravizza, et al., 2006, Ravizza, et al., 2008). This implies that the cytokine levels that we found in HS are probably not significantly different from normal controls either. This and these afore-mentioned studies also suggest that cytokine levels are not necessarily altered in chronic epilepsy. This is in accordance with our preclinical results, as we did not observe any inflammation 2 and 24 hours after amygdala kindled seizures (chapter 4). As amygdala kindling is the model that "tells us what is not necessarily lost or altered during the process of epileptogenesis" (McIntyre, 2006), it appears that inflammation is not necessarily present in the chronic epileptic state. This is supported by the fact that also in the status epilepticus model, inflammation was not always present during the chronic phase, even though animals did experience spontaneous seizures (De Simoni, et al., 2000, Lehtimaki, et al., 2003, Pernot, et al., 2011, Vezzani, et al., 2002). Moreover, the reverse is also true: inflammation does not always result in the occurrence of seizures or epilepsy. Thus the

incidence of seizures in other neurological disorders associated with brain inflammation is low and only a minority of patients develop epilepsy after incidents that are associated with inflammation such as trauma or stroke (Camilo and Goldstein, 2004, Herman, 2002, Lowenstein, 2009).
In summary, inflammatory cytokine changes have been demonstrated in both animal models and epilepsy patients, but a pro-inflammatory state is not necessarily present in the chronic epileptic state. More research is needed to identify those patients in whom inflammation contributes to epilepsy and who could benefit most from anti-inflammatory treatments. In this respect in vivo evaluation of inflammation by various imaging techniques that have been applied in other neurological disorders might offer a powerful tool for diagnostic and therapeutic purposes (Stoll and Bendszus, 2009, Wunder, et al., 2009).

NMDA-receptor alterations

Inflammatory molecules such as IL-1β can directly influence neuronal excitability by increasing the functioning of the N-methyl-D-aspartate receptor (NMDAR) through phosphorylation of this receptor (Maroso, et al., 2011, Viviani, et al., 2003). Phosphorylation of the NR2B subunit was unaltered after amygdala kindled seizures (chapter 4), which is in line with the finding that cytokine levels were not altered either. In the self-sustained limbic status epilepticus (SSLSE) model, phosphorylation increased directly after status epilepticus onset as reported previously (Huo, et al., 2006, Moussa, et al., 2001, Niimura, et al., 2005) and shortly after spontaneous seizures (chapter 5). On the contrary, phosphorylation levels decreased during epileptogenesis and 24 hours after a spontaneous seizure following SSLSE (chapter 5). Since NR2B phosphorylation facilitates its interaction with PSD-95 by anchoring the NMDAR to the post-synaptic membrane (Collingridge, et al., 2004), this reduction may indicate an altered distribution of NMDARs. Indeed, we demonstrated an increased extra-synaptic localization of NR2B during epileptogenesis. Whether this increased extra-synaptic localization is also present interictally during the chronic phase remains to be determined.
The altered membrane localization has functional implications, because extra-synaptic receptors may contribute to neuronal synchronization and neuronal cell death (Halassa, et al., 2007, Hardingham, et al., 2002, Papadia and Hardingham, 2007). Consequently, blockade of NR2B-containing NMDARs using ifenprodil reduced pyramidal cell loss in the hippocampus (chapter 5). Blockade of the extra-synaptic NMDARs or prevention of their expression might therefore offer neuroprotection against excitotoxicity after various pro-epileptogenic insults. Furthermore, targeting of these receptors might have positive effects on the development of epileptic seizures in the chronic phase. Ifenprodil has previously been shown to reduce spontaneous

epileptic activity in a mouse model of TLE (Maroso, et al., 2010). Future studies should test the antiepileptogenic properties of ifenprodil in SSLSE and other models of TLE.

Vagus nerve stimulation

Vagus Nerve Stimulation (VNS) is an adjunctive treatment for patients suffering from refractory epilepsy. Randomized controlled trials demonstrated the effectiveness of VNS in adults: seizure frequency was reduced with more than 50% in 23-39% of the VNS treated patients, compared to 13-19% of the placebo treated patients (Ben-Menachem, et al., 1994, Handforth, et al., 1998, The Vagus Nerve Stimulation Study Group, 1995). We performed the first randomized controlled trial in children, which is presented in chapter 6. We did not find a similar favourable effect as described in adults: a seizure frequency reduction of 50% or more occurred in 16% of the children in the VNS treatment group, compared to 21% in the active control group. Seizure severity was not significantly different either between these two groups. On the basis of these results the question arises whether VNS is a suitable treatment for children with refractory epilepsy.

When answering this question, we should first of all critically evaluate the outcome parameters that are used. Because seizures are the main symptom of epilepsy and the major target of its treatment, seizure frequency is most often used as the primary outcome measure. As objective evaluation by video-EEG monitoring is not feasible for long-term follow-up, seizure frequency is often registered through the use of seizure diaries. This method is the best available, but clearly results in an underestimation of seizure frequency (Hoppe, et al., 2007). Moreover, there appears to be a large discrepancy between the reported efficacy and the efficacy as perceived by patients and parents. It seems to be questionable whether the applied quantitative measures capture all the important aspects involved in epilepsy. Additionally, it should be taken into account that this patient population is highly refractory. Therefore, even a small improvement can already be relevant to the individual patient. Furthermore, VNS has a very favourable side effect profile, especially when compared to antiepileptic drugs (Ramsay, et al., 1994). Moreover, unlike the effect of antiepileptic drugs, a positive response to VNS is long-lasting (Helmers, et al., 2001, Murphy, 1999, Shahwan, et al., 2009). Therefore, all in all we believe that VNS is worthwhile considering for the individual patient. Clearly, more research on VNS that is specifically directed at children is needed. The most optimal output parameters for children should be explored as the vagus nerve has different electrophysiological properties in children (Koo, et al., 2001). Moreover, identification of predictors of clinical response would facilitate clinical practise. Furthermore, research that goes beyond the numbers like qualitative research is needed to bridge the gap between scientific evidence and clinical practise (Green and Britten, 1998).

In addition to the effects of VNS on seizure frequency, we also evaluated the influence of VNS on cytokine levels (chapter 7). The vagus nerve is involved in the so-called cholinergic anti-inflammatory reflex that inhibits production of pro-inflammatory cytokines and thereby confines inflammation (Tracey, 2007). Activation of this anti-inflammatory pathway by VNS may partly explain its anticonvulsive effects. We found an association between baseline plasma levels of IL-6 and seizure frequency reduction. However, VNS did not change interictal plasma levels of IL-1, IL-6, and IL-10. Two studies that have been performed in adult epilepsy patients did not show any influence of VNS on circulating cytokines either (Barone, et al., 2007, Majoie, et al., 2011), although levels in depressed patients increased after VNS (Corcoran, et al., 2005). Based on our results and on the current literature we cannot conclude that VNS does not have any anti-inflammatory actions in epilepsy at all. For instance, VNS might only inhibit cytokine expression in the immediate post-ictal phase. Moreover, VNS might exert local anti-inflammatory actions in the brain that may be difficult to detect in blood or CSF. Since it is impossible to test the latter hypothesis in patients, this issue should be addressed in an experimental study. As we have stated in chapter 8, such a study should apply chronic VNS in a chronic animal model for epilepsy. In this respect, the status epilepticus rather than the amygdala kindling model appears to be the most suitable, as we did not observe any inflammatory changes after amygdala kindled seizures.

Conclusion

Inflammation is not a prerequisite for the development of the epilepsy prone state in the amygdala-kindling model, or for the occurrence of hippocampal sclerosis in a cohort of temporal lobe epilepsy patients. More research is needed to identify those patients in whom inflammatory processes might actually be involved and in whom therefore anti-inflammatory treatments might be effective. Changes in the localization of the NR2B subunit do appear to play an important role in epileptogenesis and might represent a novel target for epilepsy treatments. Current treatment of children with VNS does not alter interictal cytokine levels, but cytokine profiles might be used to predict clinical response to VNS. Future studies that aim at exploring the immunomodulatory effects of VNS should use a chronic animal model for epilepsy that is associated with pro-inflammatory changes.

References

Barone, L., Colicchio, G., Policicchio, D., Di Clemente, F., Di Monaco, A., Meglio, M., Lanza, G. A., and Crea, F., 2007. Effect of vagal nerve stimulation on systemic inflammation and cardiac autonomic function in patients with refractory epilepsy. Neuroimmunomodulation 14**,** 331-336.

Ben-Menachem, E., Manon-Espaillat, R., Ristanovic, R., Wilder, B. J., Stefan, H., Mirza, W., Tarver, W. B., and Wernicke, J. F., 1994. Vagus nerve stimulation for treatment of partial seizures: 1. A controlled study of effect on seizures. First International Vagus Nerve Stimulation Study Group. Epilepsia 35**,** 616-626.

Camilo, O., and Goldstein, L. B., 2004. Seizures and epilepsy after ischemic stroke. Stroke 35**,** 1769-1775.

Collingridge, G. L., Isaac, J. T., and Wang, Y. T., 2004. Receptor trafficking and synaptic plasticity. Nat Rev Neurosci 5**,** 952-962.

Corcoran, C., Connor, T. J., O'Keane, V., and Garland, M. R., 2005. The effects of vagus nerve stimulation on pro- and anti-inflammatory cytokines in humans: a preliminary report. Neuroimmunomodulation 12**,** 307-309.

Crespel, A., Coubes, P., Rousset, M. C., Brana, C., Rougier, A., Rondouin, G., Bockaert, J., Baldy-Moulinier, M., and Lerner-Natoli, M., 2002. Inflammatory reactions in human medial temporal lobe epilepsy with hippocampal sclerosis. Brain Res 952**,** 159-169.

De Simoni, M. G., Perego, C., Ravizza, T., Moneta, D., Conti, M., Marchesi, F., De Luigi, A., Garattini, S., and Vezzani, A., 2000. Inflammatory cytokines and related genes are induced in the rat hippocampus by limbic status epilepticus. Eur J Neurosci 12**,** 2623-2633.

Green, J., and Britten, N., 1998. Qualitative research and evidence based medicine. BMJ 316**,** 1230-1232.

Halassa, M. M., Fellin, T., and Haydon, P. G., 2007. The tripartite synapse: roles for gliotransmission in health and disease. Trends Mol Med 13**,** 54-63.

Handforth, A., DeGiorgio, C. M., Schachter, S. C., Uthman, B. M., Naritoku, D. K., Tecoma, E. S., Henry, T. R., Collins, S. D., Vaughn, B. V., Gilmartin, R. C., Labar, D. R., Morris, G. L., 3rd, Salinsky, M. C., Osorio, I., Ristanovic, R. K., Labiner, D. M., Jones, J. C., Murphy, J. V., Ney, G. C., and Wheless, J. W., 1998. Vagus nerve stimulation therapy for partial-onset seizures: a randomized active-control trial. Neurology 51**,** 48-55.

Hardingham, G. E., Fukunaga, Y., and Bading, H., 2002. Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5**,** 405-414.

Helmers, S. L., Wheless, J. W., Frost, M., Gates, J., Levisohn, P., Tardo, C., Conry, J. A., Yalnizoglu, D., and Madsen, J. R., 2001. Vagus nerve stimulation therapy in pediatric patients with refractory epilepsy: retrospective study. J Child Neurol 16**,** 843-848.

Herman, S. T., 2002. Epilepsy after brain insult: targeting epileptogenesis. Neurology 59**,** S21-26.

Hoppe, C., Poepel, A., and Elger, C. E., 2007. Epilepsy: accuracy of patient seizure counts. Arch Neurol 64**,** 1595-1599.

Huo, J. Z., Dykstra, C. M., and Gurd, J. W., 2006. Increase in tyrosine phosphorylation of the NMDA receptor following the induction of status epilepticus. Neurosci Lett 401**,** 266-270.

Koo, B., Ham, S. D., Sood, S., and Tarver, B., 2001. Human vagus nerve electrophysiology: a guide to vagus nerve stimulation parameters. J Clin Neurophysiol 18**,** 429-433.

Kwan, P., and Brodie, M. J., 2000. Early identification of refractory epilepsy. N Engl J Med 342**,** 314-319.

Lehtimaki, K., Peltola, J., Koskikallio, E., Keranen, T., and Honkaniemi, J., 2003. Expression of cytokines and cytokine receptors in the rat brain after kainic acid-induced seizures. Brain Res Mol Brain Res 110**,** 253-260.

Lowenstein, D. H., 2009. Epilepsy after head injury: an overview. Epilepsia 50 Suppl 2**,** 4-9.

Majoie, H. J., Rijkers, K., Berfelo, M. W., Hulsman, J. A., Myint, A., Schwarz, M., and Vles, J. S., 2011. Vagus nerve stimulation in refractory epilepsy: effects on pro- and anti-inflammatory cytokines in peripheral blood. Neuroimmunomodulation 18**,** 52-56.

Maroso, M., Balosso, S., Ravizza, T., Liu, J., Aronica, E., Iyer, A. M., Rossetti, C., Molteni, M., Casalgrandi, M., Manfredi, A. A., Bianchi, M. E., and Vezzani, A., 2010. Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nat Med 16**,** 413-419.

Maroso, M., Balosso, S., Ravizza, T., Liu, J., Bianchi, M. E., and Vezzani, A., 2011. Interleukin-1 type 1 receptor/Toll-like receptor signalling in epilepsy: the importance of IL-1beta and high-mobility group box 1. J Intern Med 270**,** 319-326.

McIntyre, D. C., 2006. The Kindling Phenomenon. In: Pitkänen, A., Schwartzkroin, P., and Moshé, S., (Eds.), Models of seizures and epilepsy. Elsevier Academic Press, Amsterdam.

Moussa, R. C., Ikeda-Douglas, C. J., Thakur, V., Milgram, N. W., and Gurd, J. W., 2001. Seizure activity results in increased tyrosine phosphorylation of the N-methyl-D-aspartate receptor in the hippocampus. Brain Res Mol Brain Res 95**,** 36-47.

Murphy, J. V., 1999. Left vagal nerve stimulation in children with medically refractory epilepsy. The Pediatric VNS Study Group. J Pediatr 134**,** 563-566.

Niimura, M., Moussa, R., Bissoon, N., Ikeda-Douglas, C., Milgram, N. W., and Gurd, J. W., 2005. Changes in phosphorylation of the NMDA receptor in the rat hippocampus induced by status epilepticus. J Neurochem 92**,** 1377-1385.

Papadia, S., and Hardingham, G. E., 2007. The dichotomy of NMDA receptor signaling. Neuroscientist 13**,** 572-579.

Pernot, F., Heinrich, C., Barbier, L., Peinnequin, A., Carpentier, P., Dhote, F., Baille, V., Beaup, C., Depaulis, A., and Dorandeu, F., 2011. Inflammatory changes during epileptogenesis and spontaneous seizures in a mouse model of mesiotemporal lobe epilepsy. Epilepsia 52**,** 2315-2325.

Ramsay, R. E., Uthman, B. M., Augustinsson, L. E., Upton, A. R., Naritoku, D., Willis, J., Treig, T., Barolat, G., and Wernicke, J. F., 1994. Vagus nerve stimulation for treatment of partial seizures: 2. Safety, side effects, and tolerability. First International Vagus Nerve Stimulation Study Group. Epilepsia 35**,** 627-636.

Ravizza, T., Boer, K., Redeker, S., Spliet, W. G., van Rijen, P. C., Troost, D., Vezzani, A., and Aronica, E., 2006. The IL-1beta system in epilepsy-associated malformations of cortical development. Neurobiol Dis 24**,** 128-143.

Ravizza, T., Gagliardi, B., Noe, F., Boer, K., Aronica, E., and Vezzani, A., 2008. Innate and adaptive immunity during epileptogenesis and spontaneous seizures: evidence from experimental models and human temporal lobe epilepsy. Neurobiol Dis 29**,** 142-160.

Sander, J. W., 1993. Some aspects of prognosis in the epilepsies: a review. Epilepsia 34**,** 1007-1016.

Shahwan, A., Bailey, C., Maxiner, W., and Harvey, A. S., 2009. Vagus nerve stimulation for refractory epilepsy in children: More to VNS than seizure frequency reduction. Epilepsia 50**,** 1220-1228.

Stoll, G., and Bendszus, M., 2009. Imaging of inflammation in the peripheral and central nervous system by magnetic resonance imaging. Neuroscience 158**,** 1151-1160.

The Vagus Nerve Stimulation Study Group, 1995. A randomized controlled trial of chronic vagus nerve stimulation for treatment of medically intractable seizures. Neurology 45**,** 224-230.

Tracey, K. J., 2007. Physiology and immunology of the cholinergic antiinflammatory pathway. J Clin Invest 117**,** 289-296.

Vezzani, A., French, J., Bartfai, T., and Baram, T. Z., 2011. The role of inflammation in epilepsy. Nat Rev Neurol 7**,** 31-40.

Vezzani, A., Moneta, D., Richichi, C., Aliprandi, M., Burrows, S. J., Ravizza, T., Perego, C., and De Simoni, M. G., 2002. Functional role of inflammatory cytokines and antiinflammatory molecules in seizures and epileptogenesis. Epilepsia 43 Suppl 5**,** 30-35.

Viviani, B., Bartesaghi, S., Gardoni, F., Vezzani, A., Behrens, M. M., Bartfai, T., Binaglia, M., Corsini, E., Di Luca, M., Galli, C. L., and Marinovich, M., 2003. Interleukin-1beta enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. J Neurosci 23**,** 8692-8700.

Wunder, A., Klohs, J., and Dirnagl, U., 2009. Non-invasive visualization of CNS inflammation with nuclear and optical imaging. Neuroscience 158**,** 1161-1173.

Inflammation

Epilepsy is one of the most common neurological disorders. Approximately one third of all patients are resistant to current drug therapies (Kwan and Brodie, 2000, Sander, 1993). Moreover, the available drugs are mainly symptomatic and do not influence disease development or disease progression. Therefore, the development of novel antiepileptic treatments is needed. This development could be aided by elucidation of the pathophysiological mechanisms underlying epilepsy. Over the last years inflammation has been implicated as one of these possible pathophysiological mechanisms (Vezzani, et al., 2011). The clinical studies that evaluated cytokine changes in epilepsies without a prototypical inflammatory aetiology were reviewed in chapter 2 of this thesis. These studies found increased levels of interleukin (IL)-6 regardless of the tissue that was analyzed. IL-1β levels were only increased in brain specimens, whereas analyses of tumor necrosis factor alpha were inconclusive. It is unclear whether these cytokine changes are intrinsic to the epilepsy itself or whether they are related to the underlying pathology. For instance, markers of inflammation were only present in hippocampal specimens from patients with hippocampal sclerosis (HS), but not in hippocampal specimens of patients without HS (Crespel, et al., 2002, Ravizza, et al., 2008). Therefore, we evaluated whether HS influences cytokine expression by comparing a broad array of cytokines in TLE patients with and without HS (chapter 3). Levels of IL-1α and IL-1 receptor antagonist were lower in sclerotic hippocampi compared to histologically normal cortex samples from the same patients. Remarkably, we were not able to demonstrate higher levels of pro-inflammatory cytokines in HS. This suggests that the presence of HS does not determine pro-inflammatory cytokine expression. A drawback of our design was that we could not directly answer the question whether the cytokine levels in our epilepsy patients are different from basal cytokine expression, because we did not compare cytokine levels to a normal non-neurological control group. Previous studies reported similar cytokine levels in brain tissue of autopsy controls and in normal brain tissue of epilepsy patients that are similar to our controls (Ravizza, et al., 2006, Ravizza, et al., 2008). This implies that the cytokine levels that we found in HS are probably not significantly different from normal controls either. This and these afore-mentioned studies also suggest that cytokine levels are not necessarily altered in chronic epilepsy. This is in accordance with our preclinical results, as we did not observe any inflammation 2 and 24 hours after amygdala kindled seizures (chapter 4). As amygdala kindling is the model that "tells us what is not necessarily lost or altered during the process of epileptogenesis" (McIntyre, 2006), it appears that inflammation is not necessarily present in the chronic epileptic state. This is supported by the fact that also in the status epilepticus model, inflammation was not always present during the chronic phase, even though animals did experience spontaneous seizures (De Simoni, et al., 2000, Lehtimaki, et al., 2003, Pernot, et al., 2011, Vezzani, et al., 2002). Moreover, the reverse is also true: inflammation does not always result in the occurrence of seizures or epilepsy. Thus the

incidence of seizures in other neurological disorders associated with brain inflammation is low and only a minority of patients develop epilepsy after incidents that are associated with inflammation such as trauma or stroke (Camilo and Goldstein, 2004, Herman, 2002, Lowenstein, 2009).
In summary, inflammatory cytokine changes have been demonstrated in both animal models and epilepsy patients, but a pro-inflammatory state is not necessarily present in the chronic epileptic state. More research is needed to identify those patients in whom inflammation contributes to epilepsy and who could benefit most from anti-inflammatory treatments. In this respect in vivo evaluation of inflammation by various imaging techniques that have been applied in other neurological disorders might offer a powerful tool for diagnostic and therapeutic purposes (Stoll and Bendszus, 2009, Wunder, et al., 2009).

NMDA-receptor alterations

Inflammatory molecules such as IL-1β can directly influence neuronal excitability by increasing the functioning of the N-methyl-D-aspartate receptor (NMDAR) through phosphorylation of this receptor (Maroso, et al., 2011, Viviani, et al., 2003). Phosphorylation of the NR2B subunit was unaltered after amygdala kindled seizures (chapter 4), which is in line with the finding that cytokine levels were not altered either. In the self-sustained limbic status epilepticus (SSLSE) model, phosphorylation increased directly after status epilepticus onset as reported previously (Huo, et al., 2006, Moussa, et al., 2001, Niimura, et al., 2005) and shortly after spontaneous seizures (chapter 5). On the contrary, phosphorylation levels decreased during epileptogenesis and 24 hours after a spontaneous seizure following SSLSE (chapter 5). Since NR2B phosphorylation facilitates its interaction with PSD-95 by anchoring the NMDAR to the post-synaptic membrane (Collingridge, et al., 2004), this reduction may indicate an altered distribution of NMDARs. Indeed, we demonstrated an increased extra-synaptic localization of NR2B during epileptogenesis. Whether this increased extra-synaptic localization is also present interictally during the chronic phase remains to be determined.
The altered membrane localization has functional implications, because extra-synaptic receptors may contribute to neuronal synchronization and neuronal cell death (Halassa, et al., 2007, Hardingham, et al., 2002, Papadia and Hardingham, 2007). Consequently, blockade of NR2B-containing NMDARs using ifenprodil reduced pyramidal cell loss in the hippocampus (chapter 5). Blockade of the extra-synaptic NMDARs or prevention of their expression might therefore offer neuroprotection against excitotoxicity after various pro-epileptogenic insults. Furthermore, targeting of these receptors might have positive effects on the development of epileptic seizures in the chronic phase. Ifenprodil has previously been shown to reduce spontaneous

epileptic activity in a mouse model of TLE (Maroso, et al., 2010). Future studies should test the antiepileptogenic properties of ifenprodil in SSLSE and other models of TLE.

Vagus nerve stimulation

Vagus Nerve Stimulation (VNS) is an adjunctive treatment for patients suffering from refractory epilepsy. Randomized controlled trials demonstrated the effectiveness of VNS in adults: seizure frequency was reduced with more than 50% in 23-39% of the VNS treated patients, compared to 13-19% of the placebo treated patients (Ben-Menachem, et al., 1994, Handforth, et al., 1998, The Vagus Nerve Stimulation Study Group, 1995). We performed the first randomized controlled trial in children, which is presented in chapter 6. We did not find a similar favourable effect as described in adults: a seizure frequency reduction of 50% or more occurred in 16% of the children in the VNS treatment group, compared to 21% in the active control group. Seizure severity was not significantly different either between these two groups. On the basis of these results the question arises whether VNS is a suitable treatment for children with refractory epilepsy.

When answering this question, we should first of all critically evaluate the outcome parameters that are used. Because seizures are the main symptom of epilepsy and the major target of its treatment, seizure frequency is most often used as the primary outcome measure. As objective evaluation by video-EEG monitoring is not feasible for long-term follow-up, seizure frequency is often registered through the use of seizure diaries. This method is the best available, but clearly results in an underestimation of seizure frequency (Hoppe, et al., 2007). Moreover, there appears to be a large discrepancy between the reported efficacy and the efficacy as perceived by patients and parents. It seems to be questionable whether the applied quantitative measures capture all the important aspects involved in epilepsy. Additionally, it should be taken into account that this patient population is highly refractory. Therefore, even a small improvement can already be relevant to the individual patient. Furthermore, VNS has a very favourable side effect profile, especially when compared to antiepileptic drugs (Ramsay, et al., 1994). Moreover, unlike the effect of antiepileptic drugs, a positive response to VNS is long-lasting (Helmers, et al., 2001, Murphy, 1999, Shahwan, et al., 2009). Therefore, all in all we believe that VNS is worthwhile considering for the individual patient. Clearly, more research on VNS that is specifically directed at children is needed. The most optimal output parameters for children should be explored as the vagus nerve has different electrophysiological properties in children (Koo, et al., 2001). Moreover, identification of predictors of clinical response would facilitate clinical practise. Furthermore, research that goes beyond the numbers like qualitative research is needed to bridge the gap between scientific evidence and clinical practise (Green and Britten, 1998).

In addition to the effects of VNS on seizure frequency, we also evaluated the influence of VNS on cytokine levels (chapter 7). The vagus nerve is involved in the so-called cholinergic anti-inflammatory reflex that inhibits production of pro-inflammatory cytokines and thereby confines inflammation (Tracey, 2007). Activation of this anti-inflammatory pathway by VNS may partly explain its anticonvulsive effects. We found an association between baseline plasma levels of IL-6 and seizure frequency reduction. However, VNS did not change interictal plasma levels of IL-1, IL-6, and IL-10. Two studies that have been performed in adult epilepsy patients did not show any influence of VNS on circulating cytokines either (Barone, et al., 2007, Majoie, et al., 2011), although levels in depressed patients increased after VNS (Corcoran, et al., 2005). Based on our results and on the current literature we cannot conclude that VNS does not have any anti-inflammatory actions in epilepsy at all. For instance, VNS might only inhibit cytokine expression in the immediate post-ictal phase. Moreover, VNS might exert local anti-inflammatory actions in the brain that may be difficult to detect in blood or CSF. Since it is impossible to test the latter hypothesis in patients, this issue should be addressed in an experimental study. As we have stated in chapter 8, such a study should apply chronic VNS in a chronic animal model for epilepsy. In this respect, the status epilepticus rather than the amygdala kindling model appears to be the most suitable, as we did not observe any inflammatory changes after amygdala kindled seizures.

Conclusion

Inflammation is not a prerequisite for the development of the epilepsy prone state in the amygdala-kindling model, or for the occurrence of hippocampal sclerosis in a cohort of temporal lobe epilepsy patients. More research is needed to identify those patients in whom inflammatory processes might actually be involved and in whom therefore anti-inflammatory treatments might be effective. Changes in the localization of the NR2B subunit do appear to play an important role in epileptogenesis and might represent a novel target for epilepsy treatments. Current treatment of children with VNS does not alter interictal cytokine levels, but cytokine profiles might be used to predict clinical response to VNS. Future studies that aim at exploring the immunomodulatory effects of VNS should use a chronic animal model for epilepsy that is associated with pro-inflammatory changes.

References

Barone, L., Colicchio, G., Policicchio, D., Di Clemente, F., Di Monaco, A., Meglio, M., Lanza, G. A., and Crea, F., 2007. Effect of vagal nerve stimulation on systemic inflammation and cardiac autonomic function in patients with refractory epilepsy. Neuroimmunomodulation 14**,** 331-336.

Ben-Menachem, E., Manon-Espaillat, R., Ristanovic, R., Wilder, B. J., Stefan, H., Mirza, W., Tarver, W. B., and Wernicke, J. F., 1994. Vagus nerve stimulation for treatment of partial seizures: 1. A controlled study of effect on seizures. First International Vagus Nerve Stimulation Study Group. Epilepsia 35**,** 616-626.

Camilo, O., and Goldstein, L. B., 2004. Seizures and epilepsy after ischemic stroke. Stroke 35**,** 1769-1775.

Collingridge, G. L., Isaac, J. T., and Wang, Y. T., 2004. Receptor trafficking and synaptic plasticity. Nat Rev Neurosci 5**,** 952-962.

Corcoran, C., Connor, T. J., O'Keane, V., and Garland, M. R., 2005. The effects of vagus nerve stimulation on pro- and anti-inflammatory cytokines in humans: a preliminary report. Neuroimmunomodulation 12**,** 307-309.

Crespel, A., Coubes, P., Rousset, M. C., Brana, C., Rougier, A., Rondouin, G., Bockaert, J., Baldy-Moulinier, M., and Lerner-Natoli, M., 2002. Inflammatory reactions in human medial temporal lobe epilepsy with hippocampal sclerosis. Brain Res 952**,** 159-169.

De Simoni, M. G., Perego, C., Ravizza, T., Moneta, D., Conti, M., Marchesi, F., De Luigi, A., Garattini, S., and Vezzani, A., 2000. Inflammatory cytokines and related genes are induced in the rat hippocampus by limbic status epilepticus. Eur J Neurosci 12**,** 2623-2633.

Green, J., and Britten, N., 1998. Qualitative research and evidence based medicine. BMJ 316**,** 1230-1232.

Halassa, M. M., Fellin, T., and Haydon, P. G., 2007. The tripartite synapse: roles for gliotransmission in health and disease. Trends Mol Med 13**,** 54-63.

Handforth, A., DeGiorgio, C. M., Schachter, S. C., Uthman, B. M., Naritoku, D. K., Tecoma, E. S., Henry, T. R., Collins, S. D., Vaughn, B. V., Gilmartin, R. C., Labar, D. R., Morris, G. L., 3rd, Salinsky, M. C., Osorio, I., Ristanovic, R. K., Labiner, D. M., Jones, J. C., Murphy, J. V., Ney, G. C., and Wheless, J. W., 1998. Vagus nerve stimulation therapy for partial-onset seizures: a randomized active-control trial. Neurology 51**,** 48-55.

Hardingham, G. E., Fukunaga, Y., and Bading, H., 2002. Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5**,** 405-414.

Helmers, S. L., Wheless, J. W., Frost, M., Gates, J., Levisohn, P., Tardo, C., Conry, J. A., Yalnizoglu, D., and Madsen, J. R., 2001. Vagus nerve stimulation therapy in pediatric patients with refractory epilepsy: retrospective study. J Child Neurol 16**,** 843-848.

Herman, S. T., 2002. Epilepsy after brain insult: targeting epileptogenesis. Neurology 59**,** S21-26.

Hoppe, C., Poepel, A., and Elger, C. E., 2007. Epilepsy: accuracy of patient seizure counts. Arch Neurol 64**,** 1595-1599.

Huo, J. Z., Dykstra, C. M., and Gurd, J. W., 2006. Increase in tyrosine phosphorylation of the NMDA receptor following the induction of status epilepticus. Neurosci Lett 401**,** 266-270.

Koo, B., Ham, S. D., Sood, S., and Tarver, B., 2001. Human vagus nerve electrophysiology: a guide to vagus nerve stimulation parameters. J Clin Neurophysiol 18**,** 429-433.

Kwan, P., and Brodie, M. J., 2000. Early identification of refractory epilepsy. N Engl J Med 342**,** 314-319.

Lehtimaki, K., Peltola, J., Koskikallio, E., Keranen, T., and Honkaniemi, J., 2003. Expression of cytokines and cytokine receptors in the rat brain after kainic acid-induced seizures. Brain Res Mol Brain Res 110**,** 253-260.

Lowenstein, D. H., 2009. Epilepsy after head injury: an overview. Epilepsia 50 Suppl 2**,** 4-9.

Majoie, H. J., Rijkers, K., Berfelo, M. W., Hulsman, J. A., Myint, A., Schwarz, M., and Vles, J. S., 2011. Vagus nerve stimulation in refractory epilepsy: effects on pro- and anti-inflammatory cytokines in peripheral blood. Neuroimmunomodulation 18**,** 52-56.

Maroso, M., Balosso, S., Ravizza, T., Liu, J., Aronica, E., Iyer, A. M., Rossetti, C., Molteni, M., Casalgrandi, M., Manfredi, A. A., Bianchi, M. E., and Vezzani, A., 2010. Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nat Med 16**,** 413-419.

Maroso, M., Balosso, S., Ravizza, T., Liu, J., Bianchi, M. E., and Vezzani, A., 2011. Interleukin-1 type 1 receptor/Toll-like receptor signalling in epilepsy: the importance of IL-1beta and high-mobility group box 1. J Intern Med 270**,** 319-326.

McIntyre, D. C., 2006. The Kindling Phenomenon. In: Pitkänen, A., Schwartzkroin, P., and Moshé, S., (Eds.), Models of seizures and epilepsy. Elsevier Academic Press, Amsterdam.

Moussa, R. C., Ikeda-Douglas, C. J., Thakur, V., Milgram, N. W., and Gurd, J. W., 2001. Seizure activity results in increased tyrosine phosphorylation of the N-methyl-D-aspartate receptor in the hippocampus. Brain Res Mol Brain Res 95**,** 36-47.

Murphy, J. V., 1999. Left vagal nerve stimulation in children with medically refractory epilepsy. The Pediatric VNS Study Group. J Pediatr 134**,** 563-566.

Niimura, M., Moussa, R., Bissoon, N., Ikeda-Douglas, C., Milgram, N. W., and Gurd, J. W., 2005. Changes in phosphorylation of the NMDA receptor in the rat hippocampus induced by status epilepticus. J Neurochem 92**,** 1377-1385.

Papadia, S., and Hardingham, G. E., 2007. The dichotomy of NMDA receptor signaling. Neuroscientist 13**,** 572-579.

Pernot, F., Heinrich, C., Barbier, L., Peinnequin, A., Carpentier, P., Dhote, F., Baille, V., Beaup, C., Depaulis, A., and Dorandeu, F., 2011. Inflammatory changes during epileptogenesis and spontaneous seizures in a mouse model of mesiotemporal lobe epilepsy. Epilepsia 52**,** 2315-2325.

Ramsay, R. E., Uthman, B. M., Augustinsson, L. E., Upton, A. R., Naritoku, D., Willis, J., Treig, T., Barolat, G., and Wernicke, J. F., 1994. Vagus nerve stimulation for treatment of partial seizures: 2. Safety, side effects, and tolerability. First International Vagus Nerve Stimulation Study Group. Epilepsia 35**,** 627-636.

Ravizza, T., Boer, K., Redeker, S., Spliet, W. G., van Rijen, P. C., Troost, D., Vezzani, A., and Aronica, E., 2006. The IL-1beta system in epilepsy-associated malformations of cortical development. Neurobiol Dis 24**,** 128-143.

Ravizza, T., Gagliardi, B., Noe, F., Boer, K., Aronica, E., and Vezzani, A., 2008. Innate and adaptive immunity during epileptogenesis and spontaneous seizures: evidence from experimental models and human temporal lobe epilepsy. Neurobiol Dis 29**,** 142-160.

Sander, J. W., 1993. Some aspects of prognosis in the epilepsies: a review. Epilepsia 34**,** 1007-1016.

Shahwan, A., Bailey, C., Maxiner, W., and Harvey, A. S., 2009. Vagus nerve stimulation for refractory epilepsy in children: More to VNS than seizure frequency reduction. Epilepsia 50**,** 1220-1228.

Stoll, G., and Bendszus, M., 2009. Imaging of inflammation in the peripheral and central nervous system by magnetic resonance imaging. Neuroscience 158**,** 1151-1160.

The Vagus Nerve Stimulation Study Group, 1995. A randomized controlled trial of chronic vagus nerve stimulation for treatment of medically intractable seizures. Neurology 45**,** 224-230.

Tracey, K. J., 2007. Physiology and immunology of the cholinergic antiinflammatory pathway. J Clin Invest 117**,** 289-296.

Vezzani, A., French, J., Bartfai, T., and Baram, T. Z., 2011. The role of inflammation in epilepsy. Nat Rev Neurol 7**,** 31-40.

Vezzani, A., Moneta, D., Richichi, C., Aliprandi, M., Burrows, S. J., Ravizza, T., Perego, C., and De Simoni, M. G., 2002. Functional role of inflammatory cytokines and antiinflammatory molecules in seizures and epileptogenesis. Epilepsia 43 Suppl 5**,** 30-35.

Viviani, B., Bartesaghi, S., Gardoni, F., Vezzani, A., Behrens, M. M., Bartfai, T., Binaglia, M., Corsini, E., Di Luca, M., Galli, C. L., and Marinovich, M., 2003. Interleukin-1beta enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. J Neurosci 23**,** 8692-8700.

Wunder, A., Klohs, J., and Dirnagl, U., 2009. Non-invasive visualization of CNS inflammation with nuclear and optical imaging. Neuroscience 158**,** 1161-1173.

Summary

Epilepsy is one of the most common neurological disorders. The pathophysiology of epilepsy is not fully elucidated. One of the processes that might be involved in the pathophysiology of epilepsy is inflammation. In this thesis we aimed to further elucidate the role of inflammation in epilepsy and in a specific epilepsy treatment, i.e. vagus nerve stimulation (VNS).

Inflammation

In **chapter 2** we have provided an overview of all clinical studies that evaluated cytokine levels in epilepsy syndromes without a prototypical inflammatory aetiology such as temporal lobe epilepsy (TLE). Levels of interleukin (IL)-6 were increased in epilepsy patients, regardless of the kind of tissue that was analyzed. IL-1β levels were only increased in brain specimens, but not in cerebrospinal fluid or blood. Analyses of tumor necrosis factor alpha were inconclusive. It appears that these cytokine changes do not merely result from seizure activity, but that they are also related to the underlying pathology.

Therefore we evaluated whether hippocampal sclerosis, which is the most common pathology associated with TLE, was associated with cytokine changes (**chapter 3**). Using a multiplex analysis we compared the levels of a broad array of cytokines and two cytokine receptors between TLE patients with and without hippocampal sclerosis and between sclerotic hippocampus and normal cortex. We did not detect an increase in pro-inflammatory cytokines in specimens from patients with hippocampal sclerosis compared to histological normal cortex and histological normal hippocampus.

Similarly, we did not find a pro-inflammatory response in a non-neurodegenerative epilepsy model, the amygdala kindling model (**chapter 4**). We did not observe increased microglial activation in amygdala-kindled rats compared to sham controls. Moreover, cytokine levels were not altered after amygdala kindling. Phosphorylation of the NR2B subunit of the N-methyl-D-aspartate receptor (NMDAR), which is a downstream effect of cytokine signalling, was not altered either. These results imply that inflammation is not present in this model in which gross neuronal cell loss is absent. Apparently, inflammation is not a prerequisite for the epilepsy-prone state in this particular model.

Finally in **chapter 5**, we evaluated NMDAR phosphorylation in a second epilepsy model, the self-sustained limbic status epilepticus model, in which animals develop spontaneous seizures after the induction of status epilepticus. Phosphorylation of the NR2B subunit of the NMDAR increased 2 hours after the onset of status epilepticus and after the occurrence of a spontaneous seizure. On the contrary, phosphorylation levels decreased 18-24 hours after status epilepticus or a spontaneous seizure and during the intermediate epileptogenic phase. Moreover, we demonstrated that there is an increased extra-synaptic expression of the NMDAR during the epileptogenic phase. This altered localization is of importance, because the membrane localization

critically influences NMDAR functioning. Pharmacological blockade of NR2B-containing NMDARs by ifenprodil during epileptogenesis significantly reduced hippocampal cell loss, showing that NR2B-containing NMDARs contribute to excitotoxicity. Therefore, targeting of misplaced NR2B-containing NMDARs might aid neuroprotection and reduction of hyperexcitability arising after various brain injuries.

Vagus nerve stimulation

Vagus nerve stimulation is an adjunctive treatment for patients suffering from refractory epilepsy. Although the efficacy of vagus nerve stimulation has been proven in adults, no randomized controlled studies have been performed in children. Therefore, we conducted the first randomized controlled trial evaluating the effects of vagus nerve stimulation in children with refractory epilepsy, as described in **chapter 6** and **chapter 7**.

Using a double blind active controlled design, we demonstrated that the effects of vagus nerve stimulation on seizure frequency and seizure severity were not significantly different between patients that received high output (active) stimulation or low output (control) stimulation (**chapter 6**). Nonetheless, the majority of parents or guardians reported an improvement in well-being.

In addition to the above described outcome parameters, we also evaluated the effects of vagus nerve stimulation on plasma cytokine levels, while the anti-inflammatory actions of the vagus nerve might explain part of the beneficial effects of VNS (**chapter 7**). We demonstrated that VNS did not significantly alter interictal levels of IL-1β, IL-6, or IL-10. However, lower baseline plasma levels of IL-6 were associated with more seizure frequency reduction. Therefore, in the future patient selection may be aided by determination of the cytokine profile of the patient.

Finally in **chapter 8** we provided a critical appraisal of the studies on VNS in animal models for seizures and epilepsy. The mechanism of VNS is still not fully elucidated despite the large number of studies that have been conducted. Further research is therefore warranted. These future studies should apply chronic VNS in a chronic animal model for epilepsy.

Conclusions

Inflammatory changes have been demonstrated both in animal models and in epilepsy patients, but a pro-inflammatory response is not necessarily present in the chronic epileptic state. Changes in the localization of the NR2B subunit do appear to play an important role in epileptogenesis and might represent a novel target for new epilepsy treatments. Treatment of children with VNS does not alter cytokine levels, but cytokine profiles might be used to predict clinical response to VNS.

Samenvatting

Epilepsie

Epilepsie is een verzamelnaam voor verschillende ziektebeelden waarbij spontaan epileptische aanvallen optreden. Deze aanvallen kunnen zich op vele manieren uiten, bijvoorbeeld als ongecontroleerde herhaaldelijke bewegingen maar ook als een verstoring van het gevoel, bewustzijn en emoties. De aanvallen zijn vaak onvoorspelbaar en kunnen daarom verwondingen en veel stress voor de patiënt veroorzaken. Maar het hebben van epilepsie betekent meer dan alleen het hebben van aanvallen. Patiënten hebben bijvoorbeeld vaak cognitieve en psychische problemen. Daarnaast hebben medicijnen voor epilepsie veel bijwerkingen. Epilepsie kan dus leiden tot allerlei beperkingen in het werk en het dagelijks leven van de patiënt.
Het is onduidelijk hoe epilepsie precies ontstaat. Een beter begrip van de processen die leiden tot epilepsie kan bijdragen aan de ontwikkeling van nieuwe behandelingen van epilepsie en in de toekomst mogelijk aan het voorkomen van epilepsie.
In de laatste jaren zijn er steeds meer aanwijzingen dat mogelijk een ontsteking in de hersenen betrokken is bij het ontstaan en voortbestaan van epileptische aanvallen. Het doel van dit proefschrift is dan ook om verschillende aspecten van ontsteking in epilepsie nader te bestuderen. Allereerst hebben we een samenvatting gemaakt van de studies waarin tekenen van een ontsteking bij epilepsie patiënten zijn onderzocht (**hoofdstuk 2**). In **hoofdstuk 3** en **hoofdstuk 4** beschrijven we twee studies waarin we ontstekingsprocessen bij patiënten en in een diermodel voor epilepsie hebben bestudeerd. In **hoofdstuk 5** hebben we één van de processen die gerelateerd zijn aan ontsteking bestudeerd in een ander diermodel voor epilepsie. Verder hebben we gekeken naar een operatieve behandeling voor epilepsie genaamd nervus vagus stimulatie (NVS) zoals beschreven in **hoofdstuk 6** en **hoofdstuk 7**, omdat deze behandeling mogelijk invloed heeft op ontsteking. Tot slot hebben we een overzicht gemaakt van alle studies die diermodellen voor deze therapie beschreven hebben (**hoofdstuk 8**).

Ontsteking in epilepsie

Een ontsteking is een reactie van het lichaam op schade aan weefsels. Tekenen van ontsteking zijn onder andere pijn, zwelling, roodheid en warmte. Tijdens een ontstekingsreactie scheiden immuuncellen signaalstoffen uit, cytokines, die zorgen voor de communicatie tussen verschillende cellen. Een ontsteking is in principe een beschermingsreactie van het lichaam die bedoeld is om schade te beperken en te herstellen. Echter, soms kan deze reactie uit de hand lopen en juist voor extra schade zorgen. Mogelijk is er ook bij epilepsie sprake van een overmatige ontstekingsreactie, die kan bijdragen aan het ontstaan van de aanvallen.

Ontsteking bij epilepsie patiënten

In verschillende studies zijn verhoogde concentraties van cytokines aangetoond in bloed, hersenvloeistof en hersenen van patiënten. Echter, veel van deze studies hebben slechts naar één cytokine gekeken, terwijl juist de combinatie van cytokines de uitkomst van de ontstekingsreactie bepaalt. Daarnaast is niet duidelijk of de aangetoonde veranderingen het gevolg zijn van de epilepsie of van de structurele veranderingen in de hersenen die optreden bij epilepsie. Daarom hebben we gelijktijdig meerdere cytokines bepaald in hersenweefsel van patiënten die hersenchirurgie hebben ondergaan ter behandeling van epilepsie. Hierbij hebben wij patiënten met en zonder deze structurele veranderingen vergeleken. In beide groepen patiënten was de concentratie cytokines even hoog. Dit suggereert dat de structurele veranderingen in de hersenen van epilepsie patiënten geen ontsteking veroorzaken.

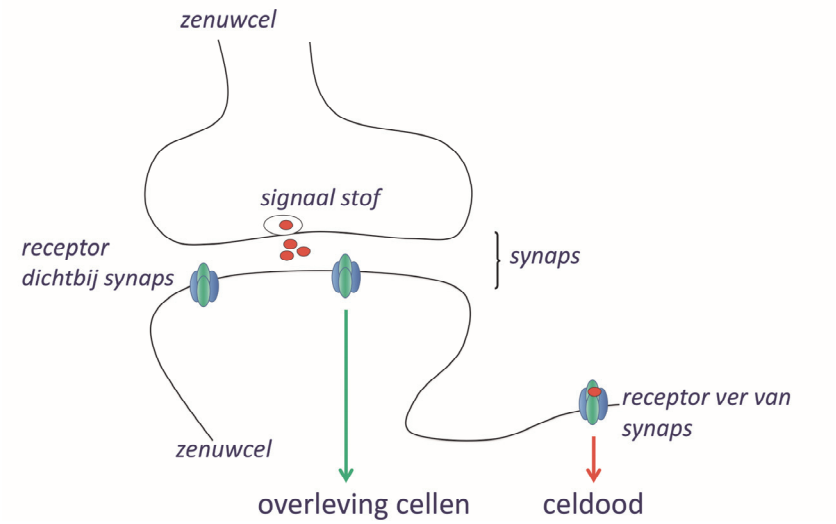
Ontsteking in diermodellen voor epilepsie

Omdat hersenweefsel alleen beschikbaar is van patiënten die in aanmerking komen voor epilepsiechirurgie, en niet van andere patiënten, hebben we ook gekeken naar ontstekingsprocessen in diermodellen voor epilepsie. Dit was al in eerdere studies gedaan, maar deze studies maakten voornamelijk gebruik van diermodellen waarin veel hersenschade optreedt. In die modellen is het daarom lastig vast te stellen of de ontsteking het gevolg is van de epilepsie of slechts van de schade die door het model veroorzaakt wordt.
We hebben daarom een diermodel voor epilepsie gebruikt waarin weinig schade optreedt om zo te kunnen bepalen of er sprake is van ontsteking in epilepsie. Allereerst hebben we gekeken naar verschillende steuncellen in de hersenen. Deze steuncellen vormen samen met de zenuwcellen de belangrijkste cellen van de hersenen. Wanneer er sprake is van ontsteking, veranderen de steuncellen van structuur en vorm. Er bleek slechts een klein verschil te zijn tussen de ratten met en zonder epileptische aanvallen. Daarnaast hebben we verschillende cytokines bepaald. Ook deze waren niet verschillend tussen de ratten met en zonder aanvallen. Concluderend kunnen we stellen dat in een diermodel voor epilepsie dat gepaard gaat met weinig zenuwcelschade, ook geen ontsteking optreedt.

Receptor veranderingen

Cytokines zouden op verschillende manieren kunnen bijdragen aan het ontstaan van aanvallen. Een van deze manieren is door de communicatie tussen zenuwcellen te beïnvloeden. Zenuwcellen communiceren met elkaar door signaalstoffen af te geven. Deze signaalstoffen passeren de ruimte tussen twee zenuwcellen, de synaps, en binden vervolgens aan een specifieke receptor. Een receptor is een eiwit op het uiteinde van een zenuwcel waaraan signaalstoffen kunnen binden, waardoor

verschillende vervolgprocessen in gang kunnen worden gezet. Een van de meest belangrijke receptoren bij epilepsie is de zogenoemde NMDA-receptor. Cytokines kunnen bijdragen aan het ontstaan van aanvallen door de werking van de NMDA-receptor te verhogen, waardoor zenuwcellen makkelijker gestimuleerd kunnen worden. Deze verandering van de NMDA-receptor trad echter niet op in het eerste diermodel dat we gebruikt hebben, mogelijk omdat we in dit model ook geen verandering in cytokines vonden.
Ook de plaats van de receptor op de zenuwcel is van belang. NMDA-receptoren dicht bij de synaps zorgen voor overleving van zenuwcellen, terwijl NMDA-receptoren die verder van de synaps liggen zorgen voor celdood (zie figuur 1). Bovendien kunnen deze laatste groep receptoren de activatie van een zenuwcel vergemakkelijken. Zowel overactivatie van zenuwcellen als celdood zijn belangrijke processen in epilepsie. Een toename van receptoren die verder weg van de synaps geplaatst zijn, zou dus kunnen bijdragen aan het ontstaan van epilepsie.



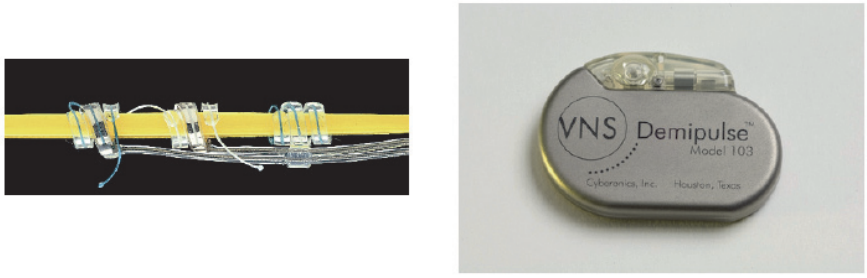
Figuur 11.1 Zenuwcellen communiceren met elkaar via signaalstoffen. Deze signaalstoffen binden aan receptoren, waarna vervolgprocessen in gang treden. Wanneer de receptor dichtbij de synaps ligt, leidt binding van de signaalstoffen tot overleving van cellen. Wanneer de receptor verder van de synaps verwijderd is, leidt binding van de signaalstoffen juist tot celdood.

Daarom hebben we de lokalisatie van de NMDA-receptor onderzocht in een tweede diermodel voor epilepsie: we hebben gekeken of de receptor dichtbij of juist ver weg van de synaps gelokaliseerd is. Met verschillende technieken hebben we aangetoond dat de lokalisatie van de receptor verandert in de periode dat epilepsie ontstaat: er zijn meer NMDA-receptoren die ver van de synaps gelokaliseerd zijn. Met andere woorden: tijdens het ontstaan van epilepsie zijn er meer receptoren die leiden tot

celdood. Deze celdood vermindert, wanneer je deze receptor met een bepaald medicijn blokkeert. Deze veranderde lokalisatie vormt dus een mogelijke verklaring voor celdood in epilepsie. Mogelijk kunnen in de toekomst specifieke therapieën ontwikkeld worden die ingrijpen op de processen die leiden tot een veranderde receptor lokalisatie.

Nervus vagus stimulatie

Ongeveer een derde van de epilepsie patiënten reageert niet goed op de huidige medicamenteuze behandeling of moet met deze behandeling stoppen vanwege ernstige bijwerkingen. Een alternatieve behandeling voor deze patiënten is hersenchirurgie. Deze ingreep heeft vaak goede resultaten, maar helaas komt maar een zeer klein deel van de patiënten hiervoor in aanmerking. Voor de overige patiënten kan NVS een geschikte behandelingsoptie zijn.
Bij NVS wordt tijdens een kortdurende operatie een elektrode rondom de nervus vagus geplaatst (figuur 2). De nervus vagus is een grote zenuw die in de nek loopt en die zorgt voor informatieoverdracht tussen de hersenen en verschillende organen. Deze elektrode wordt verbonden met een pulsgenerator die geplaatst wordt in de borst van de patiënt. Deze pulsgenerator is een apparaat dat met tussenpauzes elektrische pulsen afgeeft aan de nervus vagus.



Figuur 11.2 Links: de elektrode die rondom de nervus vagus (weergegeven door de gele draad) wordt geplaatst. Rechts: de pulsgenerator die in de borst van de patiënt wordt geïmplanteerd.

Deze behandeling kan zowel het aantal als de ernst van de aanvallen verminderen. Daarnaast kan NVS een positief effect hebben op ondermeer gedrag, stemming en kwaliteit van leven. Helaas werkt NVS slechts bij een deel van de patiënten. Op dit moment is het niet mogelijk om van te voren te voorspellen welke patiënten gunstig zullen reageren op NVS. Gerelateerd hieraan is het feit dat tot nu toe het werkingsmechanisme van NVS nog grotendeels onbekend is. Bovendien zijn er tot nu

toe nog geen studies bij kinderen verricht waarbij het effect vergeleken is met een controle behandeling.
Daarom hebben wij het effect van NVS bij kinderen geëvalueerd. In deze studie werden 41 kinderen met therapie resistente epilepsie geïmplanteerd met een nervus vagus stimulator. In het eerst deel van de studie kreeg de helft van de kinderen NVS waarbij de zenuw met dezelfde hoeveelheid stroom werd gestimuleerd als de hoeveelheid die normaal voor de behandeling gebruikt wordt. De andere helft onderging een controle behandeling waarbij ze een verwaarloosbare hoeveelheid stroom kregen. Aan het einde van de studie kregen beide groepen kinderen de gebruikelijke hoeveelheid stroom. Uit onze resultaten blijkt dat het effect van NVS niet verschilde tussen de kinderen die NVS kregen en de kinderen die de controle behandeling kregen: zowel het aantal als de ernst van de aanvallen was gelijk. Wanneer de begin periode voor de operatie werd vergeleken met het einde van de studie wanneer alle kinderen NVS kregen, bleek dat zowel het aantal als de ernst van de aanvallen verbeterde.
Naast aanvalsernst en aanvalsfrequentie, is ook een derde uitkomst van NVS geëvalueerd, namelijk het effect van NVS op cytokines. Normaal gesproken controleert de nervus vagus de omvang van een ontstekingsreactie. Mogelijk speelt deze functie ook een rol bij het werkingsmechanisme van NVS: NVS remt mogelijk de ontstekingsprocessen die bij epilepsie optreden. In de hierboven beschreven studie hebben we daarom naar ontsteking gekeken door op verschillende momenten cytokine concentraties in het bloed van de kinderen te bepalen. Er was geen effect van NVS op de cytokine concentraties. Dit zou kunnen komen doordat de verandering in cytokines te klein is om in het bloed waar te nemen. Daarnaast hebben we alleen de lange termijn effecten bekeken en kan dus niet worden uitgesloten dat NVS wel acute veranderingen in cytokine concentraties kan veroorzaken. Wel konden we op basis van de begin concentratie van een van de cytokines voorspellen of NVS het aantal aanvallen zou verminderen. Met andere woorden, op basis van de cytokine concentratie kon worden voorspeld of patiënten wel of niet op de behandeling met NVS reageerden. In de toekomst kan dit mogelijk gebruikt worden als hulpmiddel bij de selectie van patiënten die voor NVS in aanmerking komen. Voor het zo ver is, moet deze bevinding eerst bevestigd en verder geanalyseerd worden in andere studies.

Conclusie

In dit proefschrift hebben we aangetoond dat er bij bepaalde patiënten en diermodellen geen sprake is van ontsteking. De lokalisatie van een bepaalde receptor speelt een belangrijke rol bij het ontstaan van aanvallen en is daarmee mogelijk een interessant doel voor toekomstige therapieën. Tot slot blijkt dat cytokines niet direct beïnvloed worden door NVS, maar dat bepaalde cytokines wel kunnen bijdragen aan het voorspellen van het effect van NVS.

Dankwoord

Dankwoord

Aan het proefschrift dat hier voor u ligt, en de weg die hier naar toe geleid heeft, hebben veel mensen een bijdrage geleverd. Bij dezen wil ik hen allen graag bedanken. Een aantal mensen wil ik in het bijzonder noemen.

Allereerst wil ik graag mijn promotoren, prof. dr. Vles en prof. dr. De Baets, bedanken.
Lieve Hans, je bent een mentor in de breedste zin van het woord. Ik heb enorme bewondering voor je inventiviteit, toewijding en oprechtheid; bij jou draait het altijd om de inhoud. Ik heb genoten van onze samenwerking en ik hoop dat we die ook na het afronden van dit proefschrift kunnen voortzetten. Bedankt voor al het vertrouwen, niet alleen jouw vertrouwen in mij, maar ook dat ik blindelings op jou kan vertrouwen.
Beste Marc, toen ik na mijn periode in Milaan graag in Maastricht het onderzoek wilde voortzetten, heeft u ervoor gezorgd dat dit mogelijk was. Ik wil u graag bedanken voor het creëren van deze mogelijkheid en voor de ondersteuning van het onconventionele plan om te promoveren zonder mastertitel.
Ook ben ik veel dank verschuldigd aan mijn copromotor, dr. Hoogland. Lieve Govert, in de afgelopen jaren heb ik veel van je mogen leren over wetenschap, basaal onderzoek doen en moleculaire biologie in het bijzonder. Ongeacht of het weekend, 's avonds laat of een feestdag was, je was er altijd. Ik heb enorme waardering voor je betrokkenheid, niet alleen bij de experimenten op het lab, maar ook bij alle andere perikelen in het leven van een promovendus. Bedankt voor alles!
Geachte dr. Majoie, beste Marian. Jij bent de (copro)motor achter het klinische deel van dit proefschrift. Jij hebt de eerste gerandomiseerde studie naar de effecten van nervus vagus stimulatie bij kinderen geïnitieerd. Bedankt dat je me de gelegenheid hebt gegeven om deel te nemen aan deze mooie studie. Ook wil ik je graag bedanken voor je altijd enthousiaste en supersnelle reacties en feedback.
Geachte dr. Rijkers, lieve Kim. Vanaf het moment dat ik je heb leren kennen, heb ik ontzag voor de manier waarop je de rol van neurochirurg i.o., onderzoeker, begeleider, partner en moeder combineert en dat alles ook nog met een vanzelfsprekendheid die doet vergeten hoe bijzonder dit eigenlijk is. Bedankt voor alles wat je me geleerd hebt en bedankt dat je altijd achter me staat, ook op deze speciale dag.

Graag wil ik de commissieleden, prof. dr. Van Oostenbrugge, prof. dr. Ramaekers, prof. dr. Lagae, prof. dr. Drexhage, bedanken voor het beoordelen van het manuscript. Prof. dr. Drexhage, ook bedankt voor de gastvrijheid waarmee ik in uw lab ben ontvangen.
Geachte prof. dr. Temel, beste Yasin, jouw kennis en kunde op het gebied van experimentele neurochirurgie zijn indrukwekkend. Het was fijn hiervan gebruik te kunnen maken. Bedankt voor je support aan, en het meedenken met onze experimenten en natuurlijk ook voor het vrijmaken van je tijd om mijn proefschrift te beoordelen.

Geachte drs. Klinkenberg, lieve Sylvia. Jouw nooit aflatende optimisme en open houding zijn bewonderenswaardig. Ik ben je heel dankbaar voor de fijne samenwerking, waarbij je me altijd het gevoel hebt gegeven gelijkwaardig te zijn. “Een dikke merci!”
Cara dottoressa Vezzani, cara Annamaria, è stato un onore di lavorare nel suo laboratorio. Lei è un esempio per tutte le donne nella scienza. La ringrazio molto. Cara dottoressa Frasca, cara Angelisa, grazie per tutto quello che mi hai insegnato. Sono orgogliosa di essere stata coinvolta in questo bellissimo articolo. Vorrei anche ringraziare tutti gli altri dipendenti del ”lab Vezzani”, in particolare Teresa, Silvia, Mattia, Massimo e le tesiste.
Beste drs. Kessels, beste Fons, iedere (pre)klinische vraag weet jij direct in een goede statistische benadering te vertalen. Het is geruststellend te weten dat de statistiek in dit boekje jouw goedkeuring heeft. Bedankt voor alle levendige en leerzame discussies.
Mijn dank gaat ook uit naar de medewerkers van de afdeling neurochirurgie, in het bijzonder drs. Cornips, dr. Dings en drs. Schijns, zonder wiens inspanningen de klinische hoofdstukken niet tot stand hadden kunnen komen. Daarnaast wil ik ook alle patiënten en hun ouders en verzorgers bedanken die een groot deel van dit proefschrift hebben mogelijk gemaakt.
Beste prof. dr. Aldenkamp, bedankt voor het ondersteunen van mijn periode in Milaan en voor het meelezen en –schrijven met verschillende artikelen in dit proefschrift.
Beste dr. Rethans en drs. Verwijnen, beste Jan-Joost en Maarten, jullie hebben mij kennis laten maken met de kracht van kwalitatief onderzoek. Jullie passie voor onderzoek, onderwijs en de combinatie daarvan is inspirerend. Bedankt voor jullie oneindige enthousiasme en geloof in onze studie. Lieve Juul, de aanhouder wint…

Graag wil ik ook bedanken alle collega’s van de School for Mental Health and Neuroscience; Loes Leenen, voor alle hulp bij de VNbasics studie; Anne-Kathrin Theis en Natalia de Wiest, voor de hulp bij de experimenten en de analyse daarvan; Harm de Wit, voor de excellente technische ondersteuning en de fijne samenwerking; de medewerkers van het CPV, voor de zorg voor de proefdieren; de mensen van IDEE, voor het leveren van de elektrodes; de technicians, voor het verschaffen van de labfaciliteiten; Tiny Wouters, voor de mooie opmaak van het boekje; Lisa Pizzuto en Linda Linssen-Ghielen, voor het plannen van alle onmogelijke afspraken; Desiree Serpenti, voor het regelen van vele praktische zaken die nodig zijn om te kunnen promoveren; en natuurlijk mijn kamergenootjes, Nienke, Romina, Frank, Ivona, en last, but certainly not least Lukas, voor alle gezelligheid.

Omdat een substantieel deel van promotieonderzoek buiten het lab en het ziekenhuis plaats vindt, wil ik graag al mijn lieve vrienden en familie bedanken, ook hen die dit moment niet meer mee kunnen maken.
Lieve Chantal, weinig mensen snappen zó goed wat promoveren inhoudt als jij. Voor alles kan ik bij jou terecht. Bedankt dat je zo’n geweldige vriendin bent. Lieve Hermien,

ik geniet van al onze gesprekken samen. Bedankt voor je luisterend oor en je altijd nuchtere visie en advies. Lieve Mariska, na 21 jaar en heel verschillende richtingen in ons leven, vind ik het fijn dat altijd als we elkaar zien, we de draad weer oppakken waar we gebleven waren. Liebe Karo, Maastricht, Grenoble, Kopenhagen, Mailand, Utrecht oder Rotterdam; die Distanz ist nie zu groß um alles miteinander zu teilen. Vielen Dank auch für die schöne Wochenenden und das fantastische Bed & Breakfast während der Experimente. Lieve Rob, “sarcasm is a lifestyle”, dat begrijp jij als geen ander. Bedankt voor je steun en ook voor je humor, waarmee je me altijd aan het lachen weet te maken. Lieve Roos, bedankt voor alle gezelligheid en heerlijke dinertjes. Care “ragazze del residence”, Franca, Eliana, Enrica e Caterina (e Stefano, Mario, e Giuseppe), anche voi eravate lontane da casa, come me. Siete state come una famiglia ed è stato fantastico essere parte di questa famiglia. Grazie per tutto. Cara Patrizia, cara Patty, sono molto contenta che ci siamo incontrate a Milano. Grazie per la tua gentilezza e la tua cordialità, spero che resteremo amiche per molto tempo. Caro Luca, la vita in Italia non è sempre “dolce” per una nord-europea. Tu l'hai capito come nessun altro. Ti ringrazio per la tua amicizia incondizionata. Vorrei anche ringraziarti per avermi fatto conoscere tutti i tuoi amici, in particolare Federico, “il mio amico migliore”.

Lieve Jean-Paul, het allermooiste wat Maastricht mij gebracht heeft dat ben jij. Het is onbeschrijflijk fijn om mijn leven met jou te mogen delen. Bedankt voor al je geduld, steun, hulp, lievigheid en liefde. Ook dank aan je ouders voor het meeleven op afstand. Lieve Els en Willem, wat zou ik zonder jullie moeten? Jullie zijn er altijd voor me en ik kan altijd op jullie rekenen. Ik ben heel erg dankbaar dat jullie ambitie jullie naar Maastricht gebracht heeft en ik ben ontzettend trots op jullie.
Lieve mama en papa, de laatste woorden van dit dankwoord zijn voor jullie, want alles wat wij bereikt hebben, hebben we aan jullie te danken. Jullie hebben ons geleerd dat met inzet en volharding alles mogelijk is. Bedankt voor al jullie liefde en onvoorwaardelijke steun.

Curriculum Vitae

Curriculum Vitae

Marlien Aalbers werd geboren op 21 juli 1986 in Batenburg. Na het behalen van haar gymnasium diploma aan het Pax Christi College te Druten, begon ze aan de studie Geneeskunde aan de Universiteit Maastricht. Aan het einde van het eerste studiejaar startte zij met onderzoek naar motivatie van studenten voor onderwijs onder begeleiding van drs. M. Verwijnen en dr. J. Rethans. Tevens werd ze geselecteerd voor het Honoursprogramma van de opleiding Geneeskunde. In het kader van dit programma raakte zij betrokken bij het epilepsie onderzoek (prof. dr. J. Vles, prof. dr. M. de Baets, dr. K. Rijkers, dr. G. Hoogland, dr. M. Majoie). Nadat zij cum laude haar bachelor diploma Geneeskunde behaalde, is zij gestart met de master geneeskunde. Daarnaast begon zij in 2009 met promotieonderzoek naar inflammatoire processen in temporaal kwab epilepsie, waarvoor zij een Koostra Talent Fellowship ontving. Een deel van dit onderzoek werd verricht aan het Mario Negri Instituut te Milaan, onder supervisie van dr. A. Vezzani. De resultaten van dit promotieonderzoek zijn beschreven in dit proefschrift.

Marlien Aalbers was born on July 21th 1986 in Batenburg, the Netherlands. After finishing her secondary school at the Pax Christi College in Druten, she started her medical training at Maastricht University, the Netherlands. At the end of the first year of medical school, she started with a research project on students' motivation for educational activities under supervision of drs. M. Verwijnen and dr. J. Rethans. She was also selected for the Honoursprogram from the Faculty of Medicine. As part of this program, she got involved in (pre) clinical epilepsy research (prof. dr. J. Vles, prof. dr. M. de Baets, dr. K. Rijkers, dr. G. Hoogland, dr. M. Majoie). After obtaining her bachelor degree with distinction, she enrolled in the Master's of Medicine program. In 2009, she started a PhD program on inflammatory processes in temporal lobe epilepsy, for which she received a Kootstra Talent Fellowship. A part of this PhD program was carried out at the Mario Negri Institute in Milan under supervision of dr. A. Vezzani. The results of this research are described in this thesis.

List of publications

Horner's syndrome: a complication of experimental carotid artery surgery in rats.
Aalbers M*, Rijkers K*, van Winden L, Hoogland G, Vles J, Majoie M. Auton Neurosci. 2009;147:64-9

Acute seizure-suppressing effect of vagus nerve stimulation in the amygdala kindled rat.
Rijkers K*, **Aalbers M***, Hoogland G, van Winden L, Vles J, Steinbusch H, Majoie M. Brain Res. 2010;1319:155-63.

Animal models for vagus nerve stimulation in epilepsy.
Aalbers M, Vles J, Klinkenberg S, Hoogland G, Majoie M, Rijkers K. Experimental Neurology 2011;230:167-75.

Misplaced NMDA receptors in epileptogenesis contribute to excitotoxicity.
Frasca A, **Aalbers M**, Frigerio F, Fiordaliso F, Salio M, Gobbi M, Cagnotto A, Gardoni F, Battaglia G, Hoogland G, Di Luca M, Vezzani A. Neurobiology of Disease 2011;43:507-15.

Vagus nerve stimulation in children and adolescents with intractable epilepsy: a randomized controlled trial.
Klinkenberg S*, **Aalbers M***, Vles J, Cornips E, Rijkers K, Leenen L, Kessels A, Aldenkamp A, Majoie M. Developmental Medicine & Child neurology 2012;54:855-61.

The effects of vagus nerve stimulation on pro- and anti-inflammatory cytokines in children with refractory epilepsy: an exploratory study.
Aalbers M, Klinkenberg S, Rijkers K, Verschuure P, Aldenkamp A, Vles J, Majoie M. Neuroimmunomodulation 2012;19:352-358.

Rat vagus nerve stimulation model of seizure suppression: nNOS and Fos B changes in the brainstem.
Rijkers K, Majoie M, **Aalbers M**, Philippens M, Doenni V, Vles J, Steinbusch H, Moers-Hornikx V, Hopkins D, Hoogland G. J Chem Neuroanat. 2012

Behavioural and cognitive effects during vagus nerve stimulation in children with intractable epilepsy - A randomized controlled trial.
Klinkenberg S, van den Bosch C, Majoie M, **Aalbers M**, Leenen L, Hendriksen J, Cornips E, Rijkers K, Vles J, Aldenkamp A. Eur J Paediatr Neurol. 2012

* gedeeld eerste auteur

Why should I prepare? A mixed Method Study exploring the Motives of medical undergraduate Students to prepare for educational Activities
Aalbers M*, Hommes J*, Rethans J, Imbos T , Muijtjens A, Verwijnen G. Submitted

Cytokines in epilepsy: a critical review of the human literature
Aalbers M, Rijkers K, Klinkenberg S, Majoie M, De Baets M, Hoogland G, Vles J. Submitted

Chronic epilepsy without neuro-inflammation: evidence from experimental and human temporal lobe epilepsy
Aalbers M, Rijkers K, Majoie M, Dings J, Schijns O, De Baets M, Kessels A, Vles J, Hoogland G. Submitted

List of abbreviations

List of abbreviations

3-MPA	mercaptopropionic acid
AED	antiepileptic drug
AK	amygdala kindling
BBB	blood brain barrier
BSA	bovine serum albumin
CBZ	carbamazepine
CNS	central nervous system
CREB	cAMP response element-binding
CSF	cerebrospinal fluid
DAB	3,3'-diaminobenzidine
DNT	dysembryoplastic neuroepithelial tumor
DZP	diazepam
ECoG	electrocorticogram
EEG	electroencephalography
ELISA	enzyme-linked immuno sorbent assay
EM	encephalomalacia
EMG	electromyogram
eTLE	extratemporal lobe epilepsy
FBS	foetal bovine serum
FCD	focal cortical dysplasia
FLE	frontal lobe epilepsy
GAERS	genetic absence epilepsy rats from Strasbourg
GFAP	glial fibrillary acidic protein
Gp130	glycoprotein130
HMEG	hemimegalencephaly
HS	hippocampal sclerosis
IED	interictal epileptic discharges
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
IL-1RI	interleukin-1 receptor type I
IL-6R	interleukin-6 receptor
LC	locus coeruleus
LTG	lamotrigine
MCD	malformation of cortical development
MCP-1	monocyte chemotactic protein-1
MFE	multifocal epilepsy
MIP	macrophage inflammatory protein

n.a.	not applicable
NE	norepinephrine
NeuN	neuronal nuclei
NFκB	nuclear factor kappa beta
n.m.	not mentioned
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NTS	nucleus tractus solitarius
NVS	nervus vagus stimulatie
P-NR2B	phosphorylated NR2B subunit
PB	phenobarbital
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PHT	phenytoin
pPS	prolonged partial seizure
PPVT	Peabody Picture Vocabulary Test
PSD	post-synaptic density
PTZ	pentylenetetrazole
rGTCS	recurrent generalized tonic clonic seizure
RIA	radio-immunoassay
rTCS	recurrent tonic-clonic seizure
RT-PCR	reverse transcription polymerase chain reaction
SB	southern blot
SE	status epilepticus
SGTC	supependymal giant cell tumors
sGTCS	single generalized tonic-clonic seizure
SRS	spontaneous recurrent seizure
SSLSE	self-sustained limbic status epilepticus
TBS	tris buffered saline
TGFβR-I	type 1 transforming growth factor β receptor
THP-1	human acute monocytic leukemia cell line
TLE	temporal lobe epilepsy
TNF-α	tumor necrosis factor alpha
TNF-RII	TNF-α receptor type II
TSC	tuberous sclerosis complex
VNS	vagus nerve stimulation
VPA	valproate
WB	western blot
WS	West syndrome